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(21) International Application Number: PCT/US91/06569 (22) International Filing Date: 11 September 1991 (11.09.91) (30) Priority data: 581,342 12 September 1990 (12.09.90) US (60) Parent Application or Grant (63) Related by Continuation US 581,342 (CIP) Filed on 12 September 1990 (12.09.90) (71) Applicants (for all designated States except US): ZYMOGENETICS, INC. [US/US]; 4225 Roosevelt Way, N.E., Seattle, WA 98105 (US). BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West 7th Street, Austin, TX 78701 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): O'HARA, Patrick, J. [US/US]; 515 North 64th, Seattle, WA 98103 (US). HAGEN, Frederick, S. [US/US]; 1315 Lexington Way Street, Seattle, WA 98112 (US). GRANT, Francis, J. [US/US]; 7714 37th Avenue, N.E., Seattle, WA 98115 (US). MUNFORD, Robert, S. [US/US]; 5504 Greenbrier, Dallas, TX 75209 (US).		(74) Agent: PARMELEE, Steven, W.; Townsend and Townsend, One Market Plaza, 2000 Steuart Tower, San Francisco, CA 94105 (US). (81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, SD, SE, SE (European patent), SN (OAPI patent), SU*, TD (OAPI patent), TG (OAPI patent), US. Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: METHODS FOR PRODUCING ACYLOXYACYL HYDROLASE			
(57) Abstract <p>Methods are disclosed for producing acyloxyacyl hydrolase. The protein is produced from eukaryotic host cells transformed or transfected with DNA construct(s) containing information necessary to direct the expression of acyloxyacyl hydrolase. The DNA constructs generally include the following operably linked elements: a transcriptional promoter; DNA sequence encoding acyloxyacyl hydrolase, the small subunit of acyloxyacyl hydrolase or the large subunit of acyloxyacyl hydrolase; and a transcriptional terminator. In addition, isolated DNA sequences encoding acyloxyacyl hydrolase and isolated DNA sequences encoding the small or large subunit of acyloxyacyl hydrolase are disclosed.</p>			

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Description

METHODS FOR PRODUCING ACYLOXYACYL HYDROLASE

5 Technical Field

The present invention relates to DNA sequences encoding acyloxyacyl hydrolase, DNA constructs capable of directing the expression of acyloxyacyl hydrolase and methods for producing acyloxyacyl hydrolase.

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Background of the Invention

Gram-negative septicemia, the clinical consequence of gram-negative bacterial invasion into the blood stream or tissue, occurs at a frequency of between 15 71,000 and 300,000 cases annually in the United States. Approximately forty percent of septicemia cases are associated with septic shock, a serious and rapidly developing complication of septicemia. Septic shock is characterized by hypotension, oliguria, coagulation 20 defects, respiratory failure, and death.

The complex array of inflammatory responses in animals elicited by gram-negative bacteria is believed to be provoked by lipopolysaccharides (LPS) present in the outer membranes of these bacteria. Typically, an LPS 25 molecule consists of an O polysaccharide, an R-core oligosaccharide, and lipid A. The structure of lipid A is highly conserved across a wide range of bacterial genera and is generally believed to be responsible for most of the biological activities of LPS. LPS is believed to 30 provoke a number of both toxic and beneficial inflammatory responses and is believed to be responsible for the interaction of bacteria with target cells, which include macrophages, neutrophils and endothelial cells. While the toxic responses include hypotension, coagulation 35 disturbances and death, beneficial responses include enhanced antibody synthesis, mobilization of phagocytes and acute phase protein synthesis.

Presently, there are no vaccines to immunize at-risk populations against septicemia. Current treatment of septicemia relies heavily on early diagnosis followed by antibiotic therapy. Septic shock patients are treated
5 symptomatically with concurrent antibiotic therapy because of the rapid onset and severity of the symptoms associated with gram-negative septicemia. Current treatments consist of first administering a best guess antibiotic followed by
10 identification through blood cultures and adjustment of antibiotic treatment; however, such a treatment regime does not inactivate the LPS which continue to induce their toxic effects.

Immunotherapy has been suggested as a treatment for gram-negative septicemia. Ziegler et al. (New Eng. J. Med. 307: 1225-1230, 1982) conducted a randomized,
15 controlled trial using human anti-core LPS antiserum which demonstrated that immune serum reduced bacteremic mortality. The use of a human polyclonal antisera has significant drawbacks. In addition, the standardization
20 of such preparations is difficult and there is the risk of transmitting viral infections such as HIV or hepatitis. Preparations of monoclonal antibodies have been used to treat septicemia, but the efficacy of such treatment is not undisputed.

Neutrophils have been shown to contain an enzyme, acyloxyacyl hydrolase (AOAH), that partially deacylates the lipid A portion of Salmonella typhimurium LPS by removing secondary fatty acyl chains (Hall and Munford, Proc. Natl. Acad. Sci. USA 80: 6671-6675, 1983).
25 AOAH has been shown to contain two disulfide-linked subunits with apparent molecular weights of 50,000 and between 14,000-20,000 (Munford and Hall, J. Biol. Chem. 264: 15613-15619, 1989). The large subunit has been shown to be glycosylated. Munford and Hall (Science 234: 203-
30 205, 1986) showed that when rabbits were injected intradermally with AOAH-treated LPS, and subsequently challenged with an intravenous injection of untreated LPS
35

there was little or no hemorrhagic necrosis of the skin at the intradermal injection site. In contrast, rabbits that were initially injected with untreated LPS exhibited necrosis. AOA^H-treated LPS, while reducing the LPS toxicity 100-fold, was found to reduce stimulation of mitogenesis of β -lymphocytes by only a factor of 12. Munford and Hall (U.S. Patent No. 4,929,604) have suggested that AOA^H may be useful in treating/preventing septic shock caused by gram-negative bacteria.

The purification of AOA^H from neutrophils as reported by Munford and Hall (J. Biol. Chem., *ibid.*) has a number of disadvantages. Although AOA^H has been purified from a cultured human premyelocytic cell line, HL-60, and peripheral blood monocytes and neutrophils, it is only a trace protein, accounting for less than 0.001% of the protein in cell lysate. The purification method is a labor intensive, multi-step protocol that does not lend itself to commercial scale-up. A preparation of 9.5 μ g of AOA^H, accounting for 7.8% of the original activity, was purified from approximately 5×10^{11} cells grown in 150 liters of media over a period of 2 months (Munford and Hall, *ibid.*). In addition, not all HL-60 cell lines are inducible to produce AOA^H and AOA^H specific activity fluctuates 2-3 fold as the cells are passaged.

Purification of AOA^H from peripheral blood neutrophils and monocytes has the risk of co-purifying infective agents such as the hepatitis viruses, HIV-1 and other viral agents, and the availability of large amounts of blood is not always assured.

There is therefore a need in the art for a method of producing relatively large amounts of pure preparations of AOA^H, which would be useful as, inter alia, a therapeutic agent in the treatment of septic shock and for producing LPS vaccines. The present invention fulfills these and other related needs through the use of recombinant DNA technology, thus eliminating the problem

of viral contamination and providing commercially feasible quantities of biologically active recombinant AOA_H.

Summary of the Invention

5 Briefly stated, the present invention discloses isolated DNA sequences encoding acyloxyacyl hydrolase (AOA_H). In one embodiment of the invention, the DNA sequence is a cDNA sequence. Certain embodiments of the invention disclose representative DNA sequences encoding
10 AOA_H including the DNA sequence which comprises the sequence from nucleotide 354 to nucleotide 1976 of either Figures 1 or 2, the DNA sequence of Figure 3 from nucleotide 389 to nucleotide 389 to nucleotide 2011 or the DNA sequence of Figure 4 from nucleotide 120 to nucleotide
15 1742. Within other embodiments of the invention, representative DNA sequences encoding AOA_H encodes the amino acid sequence from Leucine, number 35 to Histidine, number 575 of Figures 1, 2, 3 or 4. In another embodiment of the invention, the DNA sequence further codes for the
20 amino acid sequence (R₁)_n-R₂-R₃-R₄, wherein R₁, R₂, R₃ and R₄ are lysine (lys) or arginine (arg) and n is a integer between 0 and 4.

In yet another embodiment of the invention, DNA sequences encode the small subunit of AOA_H. In certain
25 aspects the DNA sequences encoding the small subunit of AOA_H may be encoded by the DNA sequence from nucleotide 354 to nucleotide 719 of Figures 1 or 2, the DNA sequence of Figure 3 from nucleotide 389 to nucleotide 754 or the DNA sequence of Figure 4 from nucleotide 120 to nucleotide
30 485. Within other embodiments, a DNA sequence encoding the small subunit of AOA_H may encode the amino acid sequence from Leucine, number 35 to Arginine, number 154 of Figures 1, 2, 3 or 4.

Other embodiments of the invention relate to DNA
35 sequences encoding the large subunit of AOA_H. In certain embodiments of the invention, the large subunit of AOA_H may be encoded by the DNA sequence from nucleotide 720 to

nucleotide 1976 of Figures 1 or 2, the DNA sequence of Figure 3 from nucleotide 755 to nucleotide 2011 or the DNA sequence of Figure 4 from nucleotide 486 to nucleotide 1742. Other embodiments of the invention disclose a DNA
5 sequence encoding the large subunit of AOA_H comprising the amino acid sequence from Serine, number 157 to Histidine, number 575 of Figures 1, 2, 3 or 4.

In certain embodiments of the invention, DNA constructs containing information necessary to direct the
10 expression of AOA_H are disclosed. In one aspect of the invention, DNA sequences encoding secretory signal peptides are included in the DNA constructs. In certain preferred embodiments, the signal peptides are the amino acid sequences Met-Glu-Ser-Pro-Trp-Lys-Ile-Leu-Thr-Val-
15 Ala-Pro-Leu-Phe-Leu-Leu-Leu-Ser-Pro-Gly-Ala-Trp-Ala-Ser-Pro-Ala-Asn-Asp-Asp-Gln-Ser-Arg-Pro-Ser or Met-Glu-Ser-Pro-Trp-Lys-Ile-Leu-Thr-Val-Ala-Pro-Leu-Phe-Leu-Leu-Leu-Ser-Pro-Gly-Ala-Trp-Ala.

Within one embodiment of the invention,
20 eukaryotic host cells containing DNA constructs containing information necessary for the expression of AOA_H are disclosed. In another embodiment of the invention, eukaryotic host cells are transformed or transfected with a first DNA construct containing the information necessary
25 to direct the expression of the large subunit of AOA_H and a second DNA construct containing the information necessary to direct the expression of the small subunit of AOA_H. Within certain preferred embodiments, the eukaryotic host cells are cultured mammalian cells or
30 yeast cells.

In yet another embodiment of the invention, methods are described for producing the AOA_H using the eukaryotic host cells transformed or transfected with DNA constructs containing the information necessary to direct
35 the expression of AOA_H or with a first DNA construct containing the information necessary to direct the expression of the large subunit of AOA_H and a second DNA

construct containing the information necessary to direct the expression of the small subunit of AOA_H. Eukaryotic cells so transformed or transfected are then cultured under conditions conducive to expression of the AOA_H, which is then isolated from the cells or culture.

Brief Description of the Drawings

Figure 1 illustrates the nucleotide sequence and deduced amino acid sequence of a representative sequence encoding AOA_H, the C/26 AOA_H cDNA. The small arrow denotes the putative start of the small subunit. The large arrow denotes the putative start of the large subunit.

Figure 2 illustrates the nucleotide sequence and deduced amino acid sequence of a representative sequence encoding AOA_H, the 4-33 AOA_H cDNA. Symbols used are as in Figure 1.

Figure 3 illustrates the consensus AOA_H nucleotide sequence and deduced amino acid sequence. Symbols used are as in Figure 1.

Figure 4 illustrates the nucleotide sequence and deduced amino acid sequence of the AOA_H1 cDNA. Symbols used are as in Figure 1.

Figure 5 illustrates the construction of plasmid pVEG. Symbols used are T7 pro, the T7 promoter; T1 and T2, synthetic and native T7 terminators, respectively; M13, M13 intergenic region.

Figure 6 illustrates the construction of plasmid pVEG'. Symbols used are as in Figure 5 and parentheses indicate a restriction site destroyed in vector construction.

Figure 7 illustrates the construction of plasmid pVEGT'. Symbols used are as in Figure 1 and pA, the Aspergillus niger polyadenylation sequence.

Figures 8 and 9 illustrate the construction of plasmids VAPDxR and pDVEG', respectively. Symbols used are ori, the adenovirus 5 0-1 map unit sequence; E, the

SV40 enhancer; MLP, the adenovirus 2 major late promoter; L1-3, the adenovirus 2 tripartite leader; SS, a set of RNA splice sites, and pA, the SV40 polyadenylation sequence.

Figure 10, illustrates the construction of plasmid pRS431. Symbols used are SV40 prom., SV40 promoter; DHFR, the dihydrofolate reductase gene; SV40 term., SV40 polyadenylation sequence; MT-1, metallothionein-1 promoter; 4-33 AOA1 cDNA, a fragment derived from the 4-33 AOA1 cDNA clone; AOA1 cDNA, the AOA1 cDNA.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

DNA construct: A DNA molecule, or a clone of such a molecule, which has been constructed through human intervention to contain sequences arranged in a way that would not otherwise occur in nature.

Secretory Signal Sequence: A DNA sequence encoding a secretory peptide. A secretory peptide is an amino acid sequence that acts to direct the secretion of a mature polypeptide or protein from a cell. Secretory peptides are characterized by a core of hydrophobic amino acids and are typically (but not exclusively) found at the amino termini of newly synthesized proteins. Very often the secretory peptide is cleaved from the mature protein during secretion. Processing sites may be encoded within the secretory peptide or may be added to the secretory peptide by, for example, in vitro mutagenesis or ligation of a linker sequence. Certain secretory peptides may be used in concert to direct the secretion of polypeptides and proteins. One such secretory peptide that may be used in combination with other secretory peptides is the third domain of the yeast Barrier protease. As used herein, the term "secretory peptide" includes at least a functional portion of a naturally occurring secretory peptide.

Secretory peptides may or may not include a pro peptide. In general, pro peptides facilitate post-translational modifications of the proteins or target a protein to a particular organelle. As used herein, the
5 secretory pathway is understood to include the transport pathway of proteins into lysosomes and vacuoles, the transport pathway of proteins into the periplasmic space and the export pathway of proteins into the medium.

Expression Vector: A DNA construct containing
10 elements which direct the transcription and translation of DNA sequence encoding polypeptides of interest. Such elements include promoters, enhancers, transcription terminators and polyadenylation signals. By virtue of the inclusion of these elements within DNA constructs, the
15 resulting expression vectors contain the information necessary to direct the expression and/or secretion of the encoded polypeptides. Expression vectors further contain genetic information that provides for their replication in a host cell, either by autonomous replication or by
20 integration into the host genome. Examples of expression vectors commonly used for recombinant DNA are plasmids and certain viruses, although they may contain elements of both. They also may include one or more selectable markers.

25 Transfection or transformation: The process of stably and hereditably altering the genotype of a recipient cell or microorganism by the introduction of purified DNA. This is typically detected by a change in the phenotype of the recipient organism. The term
30 "transformation" is generally applied to microorganisms, while "transfection" is generally used to describe this process in cells derived from multicellular organisms.

Cultured cell: A cell capable of being grown in liquid or solid media over a number of generations. In
35 the case of cells derived from multicellular organisms, a cultured cell is a cell isolated from the organism as a single cell, a tissue, or a portion of a tissue.

As noted above, AOAH is a disulfide-linked dimer composed of a large subunit of approximately 50 kD and a small subunit of between 14 and 20 kD. As disclosed as
5 part of the present invention, AOAH is encoded by a single gene. AOAH is a trace protein making current AOAH purification procedures time consuming and expensive. (Munford and Hall, J. Biol. Chem. ibid., suggest that there are approximately 2,500 molecules per cell.) AOAH
10 removes secondary fatty acyl chains that are linked to the hydroxyl groups of the 3-hydroxytetradecanoyl residues of lipid A.

The present invention discloses representative DNA and amino acid sequences encoding AOAH. Sequences
15 encoding AOAH include those sequences resulting in minor variations in amino acid sequence, such as those due to genetic polymorphism, differences between species, and those in which blocks of amino acids have been added, deleted or replaced without substantially altering the
20 biological activity of the proteins.

In other instances one may employ such changes in the sequence of recombinant AOAH to substantially decrease or even increase the biological activity of AOAH, depending on the intended use of the preparation. The
25 term "biological activity" means the ability to remove secondary fatty acyl groups from LPS. The biological activity of AOAH may be assayed by, for example, measuring the hydrolysis of tritiated-fatty acids from ³H-acyl, ¹⁴C-glucosamine-labeled LPS as described by U.S. Patent No.
30 4,929,604, which is incorporated herein by reference. Changes in the AOAH coding sequence will result in a polypeptide sufficiently duplicative as to retain the biological activity of native AOAH.

Based on the deduced amino acid sequence of the
35 small AOAH subunit, partial homology was found between the small subunit and sphingolipid activator protein (SAP) precursor. SAPs, which are produced by the proteolytic

processing of a SAP precursor into four small subunits, are co-factors required for the activity of lysosomal hydrolases in the degradation of sphingolipids. The SAP precursor and the lysosomal hydrolases with which the SAPs work are encoded by different genes. The AOA small subunit also shows striking homology to sulfated glycoprotein 1, human pulmonary surfactant protein B and canine pulmonary surfactant protein B. The alignment of the AOA small subunit sequence with these other proteins shows that four out of the six SAP cysteines have counterparts in the AOA sequence.

Comparison of the deduced amino acid sequence of the large AOA subunit has elucidated a partial homology between the large subunit and the consensus essential serine sequence of pancreatic lipases. Pancreatic lipases have been shown to share a significant homology around the essential serine that extends 6 residues on either side of the essential serine (Mickel et al., J. Biol. Chem. 264: 12895-12901, 1989 and Lowe et al., J. Biol. Chem. 264: 20042-20046, 1989).

It is an object of the present invention to provide DNA sequences encoding acyloxyacyl hydrolase (AOA). An additional object of the present invention is to provide DNA sequences encoding the large subunit of AOA and the small subunit of AOA. It is also an object of the present invention to provide methods for producing AOA from recombinant host cells. A feature of the present invention is a DNA construct capable of directing the expression of AOA. It is a further feature of the present invention to have eukaryotic host cells containing DNA constructs capable of directing the expression of AOA. The present invention provides the advantage that AOA is produced at levels substantially higher than that found in neutrophils. In addition, the present invention provides the advantage of producing AOA that is exported from a recombinant cell into the medium where it will be more easily isolated. Thus the recombinant AOA may be

produced apart from molecules which which it is typically associated in neutrophils, thereby facilitating the preparation of substantially pure recombinant AOA_H, as discussed hereinbelow. It is a further advantage of the present invention to produce AOA_H from cultured recombinant cells, thus eliminating the risk of transmission of viral infections while providing a method for producing large amounts of biologically active AOA_H or AOA_H capable of being activated.

Although DNA sequences encoding AOA_H may be isolated from cDNA and/or genomic libraries, initial attempts by the inventors to isolate AOA_H cDNA sequences using a monoclonal antibody against AOA_H or using a mixed family of probes based on the genetic code for the AOA_H amino acid sequence were unsuccessful. The high redundancy of the genetic code for the disclosed amino acid sequence and the trace amount of AOA_H present in the cell are believed to contribute to the failure of traditional cDNA screening methods.

In one aspect of the present invention, polynucleotide sequences encoding AOA_H, and particularly DNA sequences, are isolated from amplified cDNA sequences using polymerase chain reactions (PCRs). Suitable sources from which RNA for the preparation of cDNA may be isolated include, for example, a cultured promyelocytic cell line such as HL-60 (ATCC CRL 240), a cultured lymphoma cell line such as U-937 (ATCC CRL 1593), peripheral blood monocytes or neutrophils, peripheral blood leukocytes of rabbits, chicken, pigs, mice and cows, with U-937 cells being particularly preferred.

A representative method for isolating a DNA sequence encoding AOA_H involves the use of PCR amplification. In the absence of known AOA_H DNA sequences, synthetic oligonucleotide primers were designed from an amino acid sequence derived from the amino terminal end of the large subunit of AOA_H, designated the core sequence. Due to the high redundancy of the genetic

code, the core sequence was unsuitable for designing primers for the direct amplification of AOA-encoding DNA. To overcome this problem, highly degenerate oligonucleotide primers were designed from amino-terminal and carboxy-terminal portions of the core sequence. To facilitate rescue of the amplified DNA sequence, flanking cloning sequences, such as restriction sites, were included in the primers. These degenerate primers were used to amplify AOA-encoding sequences from random-primed cDNA prepared from U-937 mRNA using the method essentially described by Lee et al. (Science 239: 1288-1291, 1988, incorporated herein by reference). The resulting amplified DNA sequence was subcloned into a cloning vector to facilitate sequence analysis of the core sequence. Suitable cloning vectors include pUC-type plasmids (Marsh et al., Gene 32: 481-485, 1984; Messing, Meth. Enzymol. 101: 21-77, 1983; Yanisch-Perron et al., Gene 33: 103, 1985). A preferred cloning vector is a bacteriophage lambda cloning vector. Particularly preferred lambda cloning vectors are λ ZAP (Stratagene Cloning Systems, La Jolla, CA) and λ HG-3 (disclosed hereinbelow).

An oligonucleotide probe corresponding to the core sequence was synthesized and used to probe a random-primed cDNA library prepared from mRNA from HL-60 cells; however, only two partial cDNA clones of approximately 800 and 900 bp were isolated out of 7.2×10^6 phage clones. Sequence analysis showed that the two clones were overlapping, but had different 5' ends, suggesting the differential splicing of messages. Sequence analysis of the clones suggested that both subunits of AOA are encoded by a single gene; however, the cDNA were incomplete and did not contain the 3' end of the AOA coding sequence.

Having obtained only partial cDNA from the λ GT11 cDNA library, PCR amplification was used to clone a complete DNA sequence encoding human AOA. Briefly, cDNA

encoding 5' and 3' AOA sequences were independently isolated from U-937 poly(A)⁺ RNA. Complementary cDNA was prepared for use as a template for the amplification of 3' AOA DNA sequences using an oligo-d(T) primer containing a cloning sequence, such as a sequence encoding various restriction sites located 5' to the oligo-d(T) tract to facilitate subcloning. It may be preferable to prepare a double-stranded cDNA as a template for amplifying 3' AOA DNA from the oligo-d(T)-primed cDNA by synthesizing a second strand using a primer encoding a sequence corresponding to a portion of the core sequence.

Double-stranded cDNA for use as a template for the amplification of 5' AOA DNA sequences was prepared from U-937 poly(A)⁺ RNA using an antisense primer encoding a sequence corresponding to a portion of the core sequence. The resulting cDNA was G-tailed using the method essentially described by Maniatis et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1982). The second strand of the G-tailed cDNA was synthesized using a poly-d(C) primer containing a cloning sequence such as a sequence encoding various restriction sites 5' to the poly-d(C) tract to facilitate subcloning.

Due to the rarity of the AOA message, the 5' and 3' templates were enriched for cDNA encoding the 5' and 3' AOA coding sequences. The cDNA preparations were first fractionated on a 1% low melt alkaline agarose gel. The lane containing the 3' cDNA was cut into 12 0.5-cm fragments, and the lane containing the 5' cDNA was cut into 8 1-cm fragments. The cDNA was eluted and amplified. The 3' AOA cDNA was amplified using a sense primer encoding a portion of the core sequence and a primer encoding a portion of the oligo-d(T) primer. The 5' AOA cDNA was amplified using a primer encoding the antisense core sequence and a primer encoding a portion of the oligo-d(C) primer. In cases where the oligo-d(T) and oligo-d(C) primers contain cloning sequences, preferred primers will encode the cloning sequences. Southern blot

analysis using the method essentially described by Maniatis et al. (ibid.) was carried out on a portion of each PCR reaction using the antisense primer as a probe. Evidence from the Southern analysis narrowed the suitable cDNA to gel fragment #3 for the amplification of 3' AOA coding sequences and fragment #4 for the amplification of 5' AOA coding sequences.

Primers for the amplification of DNA sequences encoding 5' and 3' AOA coding sequences were designed essentially as described by Hagen (copending US Patent Application Serial No. 07/320,191, which is incorporated herein by reference). Briefly, primers containing sequences termed "prime sequences" were used to facilitate the subcloning of the amplified DNA sequences, in a directional manner, into cloning vectors. Oligonucleotide primers were designed using the formula $X_nT_yN_m$, wherein X_n is a sequence of deoxynucleotide monophosphates other than deoxythymidine monophosphate (d(T)) from 3 to about 25 nucleotides in length, preferably about 12 to about 15 nucleotides in length, and T_y is one or more, preferably 2 or more, deoxythymidine monophosphates. N_m is an oligodeoxynucleotide that is the same or complementary to a terminal cDNA sequence. A 3' prime sequence, X_1T_y , was designed for use as the 3' sequence of a 3' prime primer, and a 5' prime sequence, X_2T_y , was designed for use as the 5' sequence of a 5' prime primer wherein X_1 and X_2 are as defined above, and the sequences of X_1 and X_2 are different and sufficiently noncomplementary to prevent them from annealing to each other as necessary for efficient cloning. In addition, X_1 and X_2 are non-palindromic.

Two prime primers, $X_1T_yN_1$ and $X_2T_yN_2$, were designed for the amplification of 3' AOA sequences, and two prime primers, $X_1T_yN_3$ and $X_2T_yN_4$, were designed for the amplification of 5' AOA sequences, wherein X_1 , X_2 and T_y are as defined above; N_1 is an oligodeoxynucleotide encoding a portion of the oligo-d(T) primer; N_2 is an

oligodeoxynucleotide corresponding the sense strand of the core sequence; N_3 is an oligodeoxynucleotide corresponding to the antisense strand of the core sequence; and N_4 is an oligodeoxynucleotide encoding a portion of the oligo-d(C) primer. The enriched 5' and 3' template cDNA were amplified using the prime primers essentially as described by Frohman et al. (*Proc. Natl. Acad. Sci. USA* 85: 8998-9002, 1988).

Adhesive ends were created on the amplified cDNA by treatment with T4 DNA polymerase in the presence of dATP. The treated cDNA was gel purified and subcloned into a vector containing adhesive ends complementary to the prime sequences encoded by the amplified cDNA. The DNA inserts were analyzed by restriction analysis and DNA sequence analysis. The sequence analysis confirmed that the amplified DNAs overlapped the core sequence and established a full length sequence of 2290 bp.

A full length cDNA was amplified using 5' prime primers and 3' prime primers according to the formulas $X_1T_yN_5$ and $X_2T_yN_6$ wherein X_1 , X_2 and T_y are as defined above; N_5 is a oligonucleotide sequence corresponding to the sense strand of the 5' untranslated sequence of AOA; and N_6 is an antisense oligonucleotide sequence corresponding to the 3' untranslated sequence of AOA. The primers were used to amplify the cDNA from fragment #3. The resulting PCR product was subcloned into an cloning vector as described above. The PCR inserts were analyzed by restriction analysis and DNA sequence analysis. Comparison of the full length AOA cDNA and the partial AOA cDNAs generated through PCR amplification and cDNA library screening showed that the PCR reactions incorporated mutations into the full length cDNA. These mutations were corrected by PCR amplification of fragments of template DNA having the correct sequence.

Figures 1, 2, and 3 disclose representative nucleotide sequences encoding AOA. The cDNA span 2277 bp and encode 575 residues including a 34 residue putative

pre-pro region and seven cysteines. Analysis of the deduced amino acid sequence shows a putative cleavage site between the large and small subunits at residue 36.

5 With the nucleotide and deduced amino acid sequence of human AOA_H provided herein, genomic or cDNA sequences encoding AOA_H may be obtained from libraries prepared from other mammalian species according to well known procedures. For instance, using oligonucleotide probes from human AOA_H, generally of at least about
10 fourteen nucleotides and up to twenty-five or more nucleotides in length; DNA sequences encoding AOA_H of other mammalian species, such as lagomorph, avian, bovine, porcine, murine, etc. may be obtained. If partial clones are obtained, it is necessary to join them in proper
15 reading frame to produce a full length clone, using such techniques as endonuclease cleavage, ligation and loopout mutagenesis.

A DNA sequence encoding AOA_H was inserted into a suitable expression vector, which was in turn used to
20 transfect eukaryotic cells. Expression vectors for use in carrying out the present invention will comprise a promoter capable of directing the transcription of a cloned DNA and a transcriptional terminator. The DNA sequences encoding the large and small subunits may also
25 be expressed independently either on the same or different plasmids.

To direct proteins of the present invention into the secretory pathway of the host cell, at least one secretory signal sequence is operably linked to the DNA
30 sequence of interest. Preferred secretory signals include the AOA_H secretory signal (pre-pro sequence), the alpha factor signal sequence (pre-pro sequence; Kurjan and Herskowitz, *Cell* 30: 933-943, 1982; Kurjan et al., U.S. Patent No. 4,546,082; Brake, EP 116,201, the PHO5 signal sequence (Beck et al., WO 86/00637), the BAR1 secretory
35 signal sequence (MacKay et al., U.S. Patent No. 4,613,572; MacKay, WO 87/002670), the SUC2 signal sequence (Carlson

et al., *Mol. Cell. Biol.* 3: 439-447, 1983), the α -1-antitrypsin signal sequence (Kurachi et al., *Proc. Natl. Acad. Sci. USA* 78: 6826-6830, 1981), the α -2 plasmin inhibitor signal sequence (Tone et al., *J. Biochem. (Tokyo)* 102: 1033-1042, 1987) and the tissue plasminogen activator signal sequence (Pennica et al., *Nature* 301: 214-221, 1983).

Alternatively, a secretory signal sequence may be synthesized according to the rules established, for example, by von Heinje (*Eur. J. Biochem.* 133: 17-21, 1983; *J. Mol. Biol.* 184: 99-105, 1985; *Nuc. Acids Res.* 14: 4683-4690, 1986).

Secretory signal sequences may be used singly or may be combined. For example, a first secretory signal sequence may be used singly or in combination with a sequence encoding the third domain of Barrier (described in co-pending commonly assigned U.S. Patent Application Serial No. 104,316, which is incorporated by reference herein in its entirety). The third domain of Barrier may be positioned in proper reading frame 3' of the DNA sequence of interest or 5' to the DNA sequence and in proper reading frame with both the secretory signal sequence and the DNA sequence of interest.

Host cells for use in practicing the present invention include mammalian, avian, plant, insect and fungal cells. Fungal cells, including species of yeast (e.g., *Saccharomyces* spp., *Schizosaccharomyces* spp.) or filamentous fungi (e.g., *Aspergillus* spp., *Neurospora* spp.) may be used as host cells within the present invention. Strains of the yeast *Saccharomyces cerevisiae* are particularly preferred.

Suitable yeast vectors for use in the present invention include YRp7 (Struhl et al., *Proc. Natl. Acad. Sci. USA* 76: 1035-1039, 1978), YEpl3 (Broach et al., *Gene* 8: 121-133, 1979), POT vectors (Kawasaki et al., U.S. Patent No. 4,931,373, which is incorporated by reference herein), pJDB249 and pJDB219 (Beggs, *Nature* 275: 104-106, 1978) and derivatives thereof. Such vectors will generally include

a selectable marker, which may be one of any number of genes that exhibit a dominant phenotype for which a phenotypic assay exists to enable transformants to be selected. Preferred selectable markers are those that complement host cell auxotrophy, provide antibiotic resistance or enable a cell to utilize specific carbon sources, and include LEU2 (Broach et al., *ibid.*), URA3 (Botstein et al., *Gene* 8: 17, 1979), HIS3 (Struhl et al., *ibid.*) or POT1 (Kawasaki et al., *ibid.*). Another suitable selectable marker is the CAT gene, which confers chloramphenicol resistance on yeast cells.

Preferred promoters for use in yeast include promoters from yeast glycolytic genes (Hitzeman et al., *J. Biol. Chem.* 255: 12073-12080, 1980; Alber and Kawasaki, *J. Mol. Appl. Genet.* 1: 419-434, 1982; Kawasaki, U.S. Patent No. 4,599,311) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals, Hollaender et al., (eds.), p. 355, Plenum, New York, 1982; Ammerer, Meth. Enzymol. 101: 192-201, 1983). In this regard, particularly preferred promoters are the TPII promoter (Kawasaki, U.S. Patent No. 4,599,311, 1986) and the ADH2-4^C promoter (Russell et al., *Nature* 304: 652-654, 1983; Irani and Kilgore, U.S. Patent Application Serial No. 183,130, which is incorporated herein by reference). The expression units may also include a transcriptional terminator. A preferred transcriptional terminator is the TPII terminator (Alber and Kawasaki, *ibid.*).

In addition to yeast, proteins of the present invention can be expressed in filamentous fungi, for example, strains of the fungi Aspergillus (McKnight et al., U.S. Patent No. 4,935,349, which is incorporated herein by reference). Examples of useful promoters include those derived from Aspergillus nidulans glycolytic genes, such as the ADH3 promoter (McKnight et al., *EMBO J.* 4: 2093-2099, 1985) and the ipiA promoter. An example of a suitable terminator is the ADH3 terminator (McKnight et al., *ibid.*). The expression units utilizing such

components are cloned into vectors that are capable of insertion into the chromosomal DNA of *Aspergillus*.

Techniques for transforming fungi are well known in the literature, and have been described, for instance, by Beggs (*ibid.*), Hinnen et al. (*Proc. Natl. Acad. Sci. USA* 75: 1929-1933, 1978), Yelton et al. (*Proc. Natl. Acad. Sci. USA* 81: 1740-1747, 1984), and Russell (*Nature* 301: 167-169, 1983). The genotype of the host cell will generally contain a genetic defect that is complemented by the selectable marker present on the expression vector. Choice of a particular host and selectable marker is well within the level of ordinary skill in the art.

In a preferred embodiment, a yeast host cell that contains a genetic deficiency in at least one gene required for asparagine-linked glycosylation of glycoproteins is used. Preferably, the yeast host cell contains a genetic deficiency in the *MNN9* gene (described in pending, commonly assigned U.S. Patent Application Serial Nos. 116,095 and 189,547, which are incorporated by reference herein in their entirety). Most preferably, the yeast host cell contains a disruption of the *MNN9* gene. Yeast host cells having such defects may be prepared using standard techniques of mutation and selection. Ballou et al. (*J. Biol. Chem.* 255: 5986-5991, 1980) have described the isolation of mannoprotein biosynthesis mutants that are defective in genes which affect asparagine-linked glycosylation. Briefly, mutagenized yeast cells were screened using fluoresceinated antibodies directed against the outer mannose chains present on wild-type yeast. Mutant cells that did not bind antibody were further characterized and were found to be defective in the addition of asparagine-linked oligosaccharide moieties. To optimize production of the heterologous proteins, it is preferred that the host strain carries a mutation, such as the yeast *pep4* mutation (Jones, *Genetics* 85: 22-33, 1977), which results in reduced proteolytic activity.

In addition to fungal cells, cultured mammalian cells may be used as host cells within the present invention. Preferred cultured mammalian cells for use in the present invention include the COS-1 (ATCC CRL 1650), BHK, and 293 (ATCC CRL 1573; Graham et al., *J. Gen. Virol.* 36: 59-72, 1977) cell lines. A preferred BHK cell line is the BHK 570 cell line (deposited with the American Type Culture Collection under accession number CRL 10314). In addition, a number of other mammalian cell lines may be used within the present invention, including Rat Hep I (ATCC CRL 1600), Rat Hep II (ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC CCL 75.1), Human hepatoma (ATCC HTB-52), Hep G2 (ATCC HB 8065), Mouse liver (ATCC CCL 29.1), NCTC 1469 (ATCC CCL 9.1) and DUKX cells (Urlaub and Chasin, *Proc. Natl. Acad. Sci USA* 77: 4216-4220, 1980).

Mammalian expression vectors for use in carrying out the present invention will include a promoter capable of directing the transcription of a cloned gene or cDNA. Preferred promoters include viral promoters and cellular promoters. Viral promoters include the immediate early cytomegalovirus promoter (Boshart et al., *Cell* 41: 521-530, 1985) and the SV40 promoter (Subramani et al., *Mol. Cell. Biol.* 1: 854-864, 1981). Cellular promoters include the mouse metallothionein-1 promoter (Palmiter et al., U.S. Patent No. 4,579,821), a mouse V_K promoter (Bergman et al., *Proc. Natl. Acad. Sci USA* 81: 7041-7045, 1983; Grant et al., *Nuc. Acids Res.* 15: 5496, 1987) and a mouse V_H promoter (Loh et al., *Cell* 33: 85-93, 1983). A particularly preferred promoter is the major late promoter from Adenovirus 2 (Kaufman and Sharp, *Mol. Cell. Biol.* 2: 1304-1319, 1982). Such expression vectors may also contain a set of RNA splice sites located downstream from the promoter and upstream from the DNA sequence encoding the peptide or protein of interest. Preferred RNA splice sites may be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal located downstream of the coding

sequence of interest. Polyadenylation signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, *ibid.*), the polyadenylation signal from the Adenovirus 5 E1B region and the human growth hormone gene terminator (DeNoto et al., *Nuc. Acids Res.* 9: 3719-3730, 1981). The expression vectors may include a noncoding viral leader sequence, such as the Adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites. Preferred vectors may also include enhancer sequences, such as the SV40 enhancer and the mouse μ enhancer (Gillies, *Cell* 33: 717-728, 1983). Expression vectors may also include sequences encoding the adenovirus VA RNAs.

The processing of the AOAH into the mature two chain form may be enhanced by modifying the cleavage site between the large and small subunits of AOAH to enhance cleavage of the precursor to the two-chain form. Modified cleavage sites for AOAH include amino acid sequences of the formula $(R_1)_n-R_2-R_3$, wherein R_1 through R_3 are lysine (Lys) or arginine (Arg) and n is an integer from 0 to 4, located between the large and small subunits of AOAH. Processing of AOAH by cleavage after a dibasic dipeptide such as Arg-Lys and subsequent removal of these amino acids may be enhanced by introducing the *S. cerevisiae* *KEX1* and/or *KEX2* genes into the host cell as described in pending, commonly assigned U.S. Patent Applications Serial Nos. 07/317,205; 130,370; and 144,357, and published EP 319,944 which are incorporated herein by reference. The *KEX2* gene encodes an endopeptidase that cleaves after a dibasic amino acid sequence (Fuller et al., in Leive, ed., *Microbiology*: 1986, 273-278, 1986); the expression of the *KEX1* gene (Dmochowska et al., *Cell* 50: 573-584, 1987) results in the subsequent removal of these dibasic amino acids. A DNA sequence encoding *KEX2* has been deposited with the ATCC, 12301 Parklawn Dr., Rockville, MD 20851 under accession number 67569. A cultured eukaryotic cell line transfected with one or both of these genes is thus useful

for expressing AOA having a modified cleavage site between the large and small subunits. Processing sites may be inserted between the sequences encoding the large and small subunits by, for example, in vitro mutagenesis.

5 Cloned DNA sequences may be introduced into cultured mammalian cells by, for example, calcium phosphate-mediated transfection (Wigler et al., *Cell* 14: 725, 1978; Corsaro and Pearson, *Somatic Cell Genetics* 7: 603, 1981; Graham and Van der Eb, *Virology* 52: 456, 1973.) Other
10 techniques for introducing cloned DNA sequences into mammalian cells, such as electroporation (Neumann et al., *EMBOJ.* 1: 841-845, 1982), may also be used. In order to identify cells that have integrated the cloned DNA, a selectable marker is generally introduced into the cells
15 along with the gene or cDNA of interest. Preferred selectable markers for use in cultured mammalian cells include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. A
20 preferred amplifiable selectable marker is the DHFR gene. Selectable markers are reviewed by Thilly (*Mammalian Cell Technology*, Butterworth Publishers, Stoneham, MA, which is incorporated herein by reference). The choice of selectable markers is well within the level of ordinary
25 skill in the art.

 Selectable markers may be introduced into the cell on a separate plasmid at the same time as the gene of interest, or they may be introduced on the same plasmid. If on the same plasmid, the selectable marker and the gene
30 of interest may be under the control of different promoters or the same promoter, the latter arrangement producing a dicistronic message. Constructs of this type are known in the art (for example, Levinson and Simonsen, U.S. Patent No. 4,713,339). It may also be advantageous
35 to add additional DNA, known as "carrier DNA" to the mixture which is introduced into the cells.

Transfected mammalian cells are allowed to grow for a period of time, typically 1-2 days, to begin expressing the DNA sequence(s) of interest. Drug selection is then applied to select for growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an amplifiable selectable marker the drug concentration may be increased in a stepwise manner to select for increased copy number of the cloned sequences, thereby increasing expression levels.

Promoters, terminators and methods for introducing expression vectors encoding AOAH into plant, avian and insect cells are well known in the art. The use of baculoviruses, for example, as vectors for expressing heterologous DNA sequences in insect cells has been reviewed by Atkinson et al. (Pestic. Sci. 22: 215-224, 1990). The use of Agrobacterium rhizogenes as vectors for expressing genes in plant cells has been reviewed by Sinkar et al. (J. Biosci. (Bangalore) 11: 47-58, 1987).

Host cells containing DNA constructs of the present invention are then cultured to produce AOAH. The cells are cultured according to standard methods in a culture medium containing nutrients required for growth of mammalian or yeast host cells. A variety of suitable media are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins, minerals and growth factors. The growth medium will generally select for cells containing the DNA construct by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker on the DNA construct or co-transfected with the DNA construct.

Yeast cells, for example, are preferably cultured in a chemically defined medium, comprising a non-amino acid nitrogen source, inorganic salts, vitamins and essential amino acid supplements. The pH of the medium is preferably maintained at a pH greater than 2 and less than

8, preferably at pH 6.5. Methods for maintaining a stable pH include buffering and constant pH control, preferably through the addition of sodium hydroxide. Preferred buffering agents include succinic acid and Bis-Tris (Sigma Chemical Co., St. Louis, MO). Yeast cells having a defect in a gene required for asparagine-linked glycosylation are preferably grown in a medium containing an osmotic stabilizer. A preferred osmotic stabilizer is sorbitol supplemented into the medium at a concentration between 0.1 M and 1.5 M., preferably at 0.5 M or 1.0 M. Cultured mammalian cells are generally cultured in commercially available serum-containing or serum-free media. Selection of a medium appropriate for the particular cell line used is within the level of ordinary skill in the art.

The AOA^H produced according to the present invention may be purified by affinity chromatography on an antibody column using antibodies directed against AOA^H. Additional purification may be achieved by conventional chemical purification means, such as liquid chromatography, gradient centrifugation, and gel electrophoresis, among others. Methods of protein purification are known in the art (see generally, Scopes, R., Protein Purification, Springer-Verlag, NY (1982), which is incorporated herein by reference) and may be applied to the purification of the recombinant AOA^H described herein; see also a purification protocol described in U.S. 4,929,604. Substantially pure recombinant AOA^H of at least about 50% is preferred, at least about 70-80% more preferred, and 95-99% or more homogeneity most preferred, particularly for pharmaceutical uses. Once purified, partially or to homogeneity, as desired, the recombinant AOA^H may then be used therapeutically.

The recombinant AOA^H molecules of the present invention and pharmaceutical compositions thereof are useful for administration to mammals, including humans, to treat a variety of conditions associated with the toxicity

of gram-negative bacterial infections. For instance, although a gram-negative bacterial infection can itself be treated with conventional antibiotics, the AOA_H preparations described herein may be used to treat or
5 prevent the LPS toxicity associated with such infections such as disseminated intravascular coagulation and others as described above.

The pharmaceutical compositions are intended for parenteral, topical, oral or local administration for
10 prophylactic and/or therapeutic treatment. Preferably, the pharmaceutical compositions are administered parenterally, i.e., intravenously, subcutaneously, or intramuscularly. Thus, this invention provides compositions for parenteral administration which comprise
15 a solution of the recombinant AOA_H molecules dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine, 20-30% glycerol and the like. These compositions may be sterilized by
20 conventional, well known sterilization techniques. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The
25 compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium
30 chloride, potassium chloride, calcium chloride, etc. The concentration of recombinant AOA_H in these formulations can vary widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily by fluid volumes,
35 viscosities, etc., in accordance with the particular mode of administration selected. Actual methods for preparing parenterally administrable compounds will be known or

apparent to those skilled in the art and are described in more detail in for example, Remington's Pharmaceutical Science, 16th ed., Mack Publishing Company, Easton, PA (1982), which is incorporated herein by reference.

5 The compositions containing the recombinant AOA molecules can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a gram-negative bacterial disease in an amount sufficient to cure or at least partially arrest the effects of LPS toxicity associated with the disease and its complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on the severity of the gram-negative infection and the general state of the patient but generally range from about 1 μ g to about 10 mg of recombinant AOA per 70 kg of body weight. It must be kept in mind that the materials of the present invention may generally be employed in serious bacterial disease states, that is, life-threatening or potentially life threatening situations. In such cases, in view of the minimization of extraneous substances and the specificity of the recombinant AOA made feasible by this invention, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these recombinant AOA compositions.

 In prophylactic applications, compositions containing the recombinant AOA are administered to a patient susceptible to or otherwise at risk of a gram-negative disease to enhance the patient's own anti-bacterial/anti-LPS capabilities. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts will again depend on the patient's state of health.

35 Single or multiple administrations of the compositions can be carried out with the dose levels and pattern being selected by the treating physician. In any

event, the pharmaceutical formulations should provide a quantity of recombinant AOAH of this invention sufficient to effectively treat the patient.

To summarize the examples which follow, Example 1 describes the construction of cloning and expression vectors. Example 2 describes cloning of DNA sequences encoding human AOAH. Example 3 describes the expression of AOAH in mammalian cells.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Restriction endonucleases and other DNA modification enzymes (e.g., T4 polynucleotide kinase, calf alkaline phosphatase, DNA polymerase I (Klenow fragment), T4 polynucleotide ligase) were obtained from Boehringer Mannheim Biochemicals, Bethesda Research Laboratories (BRL) and New England Biolabs and were used as directed by the manufacturer, unless otherwise noted.

Oligonucleotides were synthesized on an Applied Biosystems Model 380A DNA synthesizer and purified by polyacrylamide gel electrophoresis on denaturing gels. *E. coli* cells were transformed as described by Maniatis et al. (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1982, incorporated by reference herein) or as described by Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Second Edition, 1988, incorporated by reference herein). M13 and pUC cloning vectors and host strains were obtained from BRL.

Example 1

Construction of Cloning and Expression Vectors

A. Construction of pVEG

To permit transcription of cloned cDNA without prior endonuclease digestion, bacteriophage T7 transcriptional terminators were added to a cloning vector. The sequence of the putative T7 RNA transcription

terminator, which lies between gene 10 and gene 11 of bacteriophage T7, is disclosed by Dunn and Studier (*J. Mol. Biol.* 166: 477-536, 1983). As shown in Figure 5, four synthetic oligonucleotides were designed from this sequence and ligated into the vector pGEM-1 (obtained from Promega Biotec, Madison, WI), a plasmid containing a bacterial origin of replication, ampicillin resistance gene, and the T7 promoter adjacent to a multiple cloning site. Terminal phosphates were added to the 5' ends of oligonucleotides ZC776 and ZC777 with T4 polynucleotide kinase and ATP, under standard conditions (Maniatis et al. *ibid*). (The sequences of these and other oligonucleotides referred to herein are shown in Table 1.) After the incubation, the kinase was heat killed at 65°C for 10 min. Twenty-five nanograms of oligonucleotide ZC775 and 25 ng of oligonucleotide ZC776 were annealed by incubation at 65°C for 15 minutes, then allowed to cool to room temperature in 500 ml of water. Oligonucleotides ZC777 and ZC778 were similarly annealed. The annealed oligonucleotides were stored at -20°C until use. The vector pGEM-1 was digested with Pst I and Hind III, and the linearized vector DNA was purified by agarose gel electrophoresis. The synthetic T7 terminator (annealed oligonucleotides ZC775, ZC776, ZC777 and ZC778) was then cloned into pGEM-1. Twenty-five nanograms of vector plus an equal molar amount of each of the annealed oligonucleotides ZC775/ZC776 and ZC777/ZC778 were combined in a 10 µl reaction mix. After an overnight ligation at 14°C, the DNA was transformed into competent *E. coli* JM83 cells, and the transformed cells were selected for ampicillin resistance. Plasmid DNA was prepared from selected transformants by the alkaline lysis procedure (Birnboim and Doly, *Nuc. Acids Res.* 7: 1513-1523, 1979). A portion of the DNA from these samples was cut with Pst I and Hind III and analyzed on a 4% polyacrylamide gel to identify clones that released an 80 bp Pst I-Hind III fragment. Other diagnostic cuts, such as Eco RI and Not

I, were also made. One of the isolates, designated pGEMT, was shown by restriction analysis to contain the T7 terminator fragment.

		<u>Table 1</u>	
		<u>Oligonucleotide</u>	<u>Sequence (5' - 3')</u>
5		ZC525	GGA ATT CT
		ZC526	GAT CAG AAT TCC
		ZC553	AAT TGA TAG CGG CCG CTT ACT GCA
10		ZC554	GTA AGC GGC CGC TAT C
		ZC775	GCT AGC ATA ACC CCT TGG GGC CTC TAA ACG GGT CT
		ZC776	CTC AAG ACC CGT TTA GAG GCC CCA AGG GGT TAT GCT AGC TGC A
15		ZC777	TGA GGG GTT TTT TGC TGA AAG GAG GAA CTA TGC GGC CGC A
		ZC778	AGC TTG CGG CCG CAT AGT TCC TCC TTT CAG CAA AAA ACC C
		ZC1750	AGG GAG ACC GGA ATT CCC CCC CCC C
20		ZC1751	AAT TCT GTG CTC TGT CAA C
		ZC1752	GAT CCT TGA CAG AGC ACA G
		ZC2063	GAT CCA AAC TAG TAA AAG AGC T
		ZC2064	CTT TTA CTA GTT TG
		ZC2465	ACA GAC TGT TCC ATA GCT AAT TTA ATT TTC TGG CAG AT
	25		ZC2487
		ZC2488	GAC TCG AGT CGA CAT CGA TCA GCC CCC CCC CC
30			ZC2489
		ZC2631	AGG GAG ACC GGA ATT CCC ATG GAA CAG TCT GTG CCA TTC AAA GAT G
		ZC2632	GAC AGA GCA CAG AAT TCG ACT CGA GTC GAC ATC GAT CAG
35		ZC2633	AGG GAG ACC GGA ATT CGA CTC GAG TCG ACA TCG ATC AG

Table 1 continued

	ZC2703	GAC AGA GCA CAG AAT TCG AGC ACA CAG CAT TGC ACA GTC GT
5	ZC2704	AGG GAG ACC GGA ATT CTC CAG CTC TTT GTG TGT GGC TCT C
	ZC3074	ACT TGG GAA TTC GTC GAC CAC CAT GCA GTC CCC CTG GAA A
	ZC3075	TTT ACA AAA CTC GAG AGT GTG
10	ZC3076	CAC ACT CTC GAG TTT TGT AAA CAG AAC ACT GG
	ZC3077	AAC ATG GGA TCC ATT GGG CAG GTG GGA ATT TAG ATG CTT CAG AGT CTG CAT GAC
	ZC3078	CCC AAT GGA TCC CAT GTT ATT TTG TAT GGC TTA CCA GAT
15	ZC3079	GGT GCA TGG TCG ACG AAT TCT CAG TGC CCG CCT
	ZC3202	TTA ATT TTC TGG CAG ATC TTG GCC
	ZC3203	TAG GGT GTG TAC TAG TGG TGT CTG

20

The native T7 terminator from plasmid pAR2529 (Rosenberg et al., Gene 56: 125-135, 1987) was added to plasmid pGEMT. Plasmid pGEMT was digested with Bam HI and plasmid pAR2529 was digested with Bam HI and Bgl II (Figure 5). The Bam HI-Bgl II terminator fragment from pAR2529 was purified by agarose gel electrophoresis. The terminator fragment was ligated to Bam HI digested pGEMT, and the DNA was transformed into competent *E. coli* LM1035 cells. Colonies that were ampicillin resistant were inoculated into 5 ml cultures for overnight growth. Plasmid DNA prepared by the alkaline lysis procedure was screened for proper terminator orientation by Bam HI-Sal I digestion and electrophoresis on an 8% polyacrylamide gel. A clone that contained the terminator in the correct orientation, as evidenced by the presence of a 130 bp Bam HI-Sal I fragment, was chosen and named pGEMTT (Figure 5).

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To allow pGEMTT to be packaged as single-stranded DNA in the presence of M13 phage proteins, the M13 intergenic region from pUC382 (similar to pUC118 and 119 as disclosed by Vieira and Messing, *Methods Enzymol.* 153: 3-11, 1987) was added to pGEMTT (Figure 5). Plasmid pGEMTT was digested with Fsp I and Nar I, and the fragment containing the T7 promoter and transcription terminator was purified. Plasmid pUC382 was digested with Fsp I and Nar I, and the fragment encoding the ampicillin resistance gene and the M13 intergenic region was gel purified. These fragments were then ligated together in the presence of T4 DNA ligase. The ligated DNA was transformed into competent *E. coli* LM1035 cells. Plasmid DNA from twelve ampicillin-resistant colonies was prepared by the alkaline lysis method, and the DNA was screened by digestion with Ava I. The appropriate construction gave two bands, one of 2430 bp and another of 709 bp. One such isolate was chosen and named pVEG.

20 B. Construction of pVEGT'

Synthetic oligonucleotides encoding the prime sequence were added to pVEG between the Bam HI and Eco RI sites (Figure 6). Plasmid pVEG was digested with Bam HI and Eco RI and the vector fragment was gel purified. Ninety-six nanograms each of oligonucleotides ZC1751 and ZC1752 were annealed in 4.5 μ l of 10 mM Tris pH 7.5, 20 mM MgCl₂ and 10 mM NaCl at 65°C for 20 minutes, then the mixture was cooled to room temperature over a period of 30 minutes. The annealed oligonucleotides were ligated to the pVEG vector fragment with T4 DNA ligase and then transformed into competent *E. coli* LM1035 cells. After growing overnight to develop the colonies, a filter lift was taken of the colonies on the agar plate. The filter was probed with ³²P-labeled oligonucleotide ZC1751. All of the colonies were positive. Plasmid DNA was prepared from cultures grown from 12 of the colonies. The plasmid DNA was screened by digestion with Sst I to verify the

absence of the Sst I site between the Eco RI and Bam HI sites of pVEG. All 12 of the plasmid DNAs were negative for Sst I digestion. One of these 12 isolates was chosen and named pVEG'.

5 A polyadenylation sequence derived from an *Aspergillus* alcohol dehydrogenase cDNA was added to pVEG. As shown in Figure 7, plasmid pM098 (disclosed in published European patent application EP 272,277 and deposited with American Type Culture Collection under accession number 10 53428) was digested with Dra I and Bam HI, and the approximately 150 bp poly(A) fragment was purified by agarose gel electrophoresis. This fragment contained mostly poly(A) sequence with very little flanking cDNA. To clone the poly(A) cDNA fragment into pVEG, pVEG was 15 digested with Bam HI and Sma I, and the 3.4 kb vector fragment was gel purified. The vector and poly(A) fragments were ligated together with T4 DNA ligase to produce vector pVEGT (Figure 7).

Synthetic oligonucleotides encoding the prime 20 sequence were added to pVEGT. To accomplish this, pVEGT was digested with Not I and Sst I, and the 370 bp fragment containing the poly(A) sequence and the two T7 transcriptional terminators was purified by agarose gel electrophoresis. Plasmid pVEG' was digested with Not I 25 and Bam HI, and the 3.2 kb vector fragment was gel-purified. Two oligonucleotides (ZC2063 and ZC2064) that formed, when annealed, a Bam HI-Sst I adapter were synthesized. The two oligonucleotides were individually 30 kinased and annealed, and ligated with the linearized vector and the poly(A)-terminator fragment. The resultant vector, designated pVEGT' (Figure 3), contained a T7 RNA transcription promoter, an Eco RI cloning site flanked by the prime sequence, a poly(A) tract, and two T7 RNA polymerase terminators.

C. Construction of DVEG

The mammalian expression vector pVAPDBam8 (Figure 8), an adenovirus-based vector, was the starting material for the construction of a mammalian cell vector containing the directional cloning features. The important elements of this vector are an adenovirus origin of replication, an SV40 enhancer, the adenovirus 2 major late promoter and tripartite leader sequence, a pair of RNA splice sites, a cloning site, and a poly(A) addition sequence. As will be appreciated by those familiar with the art, these elements may be obtained from a variety of sources, and the particular starting materials and manipulations described herein were chosen for convenience. To facilitate the subcloning of Eco RI cloned cDNA into this expression vector, an Eco RI cloning site was added to pVAPDBam8.

The vector was first modified so that the prime sequence could be inserted at the Bcl I site. To prepare pVAPDBam8 for digestion with Bcl I, which requires the absence of methylated sites within the recognition sequence, the plasmid was transformed into *E. coli* DH1 (a modification plus and restriction minus strain) and subsequently transformed into *E. coli* GM-48, (a modification minus and restriction minus). The resulting plasmid, pVAPDBam8-1, was digested with Bcl I. An adapter formed by two kinased, annealed oligonucleotides, ZC525 and ZC526, was ligated with the Bcl I-digested vector. To make this construction two adapters had to blunt-end ligate, then the double adapter had to ligate into the Bcl I cloning site, resulting in a vector having two Eco RI sites flanked by Bcl I sites. This vector was named VAPDR (Figure 8). To remove the other Eco RI site near the viral origin of replication of this vector, VAPDR was digested with Xho I and Pvu I, and the 3.2 kb fragment, containing the splice sites and polyadenylation sequence, was gel purified. From the similar vector pDX (disclosed in published European patent application EP 276,846 and

shown in Figure 8), a 1.7 kb Xho I-Pvu I fragment, containing the adenovirus origin of replication, SV40 enhancer and adenovirus major late promoter, was gel purified. These two fragments were ligated together with T4 DNA ligase to produce the vector VAPDxR (Figure 8).

The M13 intergenic region was then added to VAPDxR. Plasmid pVEG was digested with Pvu II and Nar I, blunted with T4 DNA polymerase, then Bam HI linkers were added with T4 DNA ligase. The ligation products were digested with Bam HI, and the DNA fragment was gel purified. VAPDxR was digested to completion with Bam HI and partially digested with Bcl I. The Bcl I-Bam HI fragment containing the adenovirus expression unit was gel purified. The fragments from pVEG and VAPDxR were ligated and transformed into competent LM1035 cells. The construction was screened for correct orientation of the intergenic region. The desired orientation would provide single-stranded DNA of anti-sense polarity in regard to RNA synthesized by the major late promoter. A construct having this configuration was named pDVEG (Figure 9).

To add the prime sequence to pDVEG, this plasmid was digested with Eco RI (Figure 9). The prime sequence, constructed by annealing oligonucleotides ZC1773 and ZC1774, was ligated to the Eco RI-digested vector. The ligated DNA was electroporated into DH5 α F'^{Tr} cells (obtained from Bethesda Research Laboratories), and the cells were plated. Colony blots were obtained and probed with labeled ZC1773 and ZC1774. Plasmid DNA was prepared from positive colonies and electroporated into XL-I blue cells (obtained from Stratagene Cloning Systems), which contain a tetracycline resistant F' required for M13 infection. Plasmid DNA was prepared from colonies that were resistant to both tetracycline and ampicillin. The region around the Eco RI site was sequenced by double-stranded dideoxy-chain termination DNA sequence analysis. A construct with the correct orientation of the prime sequence was selected and named pDVEG'.

D. Construction of λ HG3

Plasmid pGEMT was subcloned into λ GT11 to facilitate rescue and analysis of cloned DNA sequences as follows. Lambda GT11 DNA was digested with Eco RI and the terminal phosphates were removed by treatment with calf alkaline phosphatase. Plasmid VAPxR (Example 1D) was digested with Pst I and was gel-purified.

Oligonucleotides ZC554 and ZC553 were designed to form, when annealed, an Eco RI-Pst I adapter with an internal Not I site. Oligonucleotides ZC554 and ZC553 were kinased and annealed and ligated to the linearized VAPDxR vector. The ligation product was gel purified and ligated to the Eco RI-digested lambda GT11. The ligation mixture was packaged and plated on *E. coli* Y1088 cells. Phage containing the VAPDxR vector were plaque-purified using nick-translated, gel-purified, VAPDxR DNA containing the Eco RI-Pst I adapter. One such isolate was called λ GH4. Plasmid pGEMT was inserted into the Not I site of λ GH4. Lambda GH4 was linearized by digestion with Not I and treated with calf alkaline phosphatase. Plasmid pGEMT (Example 1A) was linearized by digestion with Not I. The linearized- λ GH4 and linearized pGEMT were ligated together, packaged, and plated on *E. coli* Y1088 cells. A clone containing pGEMT was plaque-purified using nick-translated pGEMT as a probe. The construction was verified by digestion with Not I and was designated λ HG3.

E. Construction of the Mammalian Expression Vector

Zem229R

The vector Zem229R was constructed as shown in Figure 10 from Zem229. Plasmid Zem229 is a pUC18-based expression vector containing a unique Bam HI site for insertion of cloned DNA between the mouse metallothionein-1 promoter and SV40 transcription terminator and an expression unit containing the SV40 early promoter, mouse dihydrofolate reductase gene, and SV40 terminator. Zem229

was modified to delete the two Eco RI sites by partial digestion with Eco RI, blunting with DNA polymerase I (Klenow fragment) and dNTPs, and re-ligation. Digestion of the resulting plasmid with Bam HI followed by ligation of the linearized plasmid with Bam HI-Eco RI adapters resulted in a unique Eco RI cloning site. The resultant plasmid was designated Zem229R.

Example 2

10 The Cloning of DNA Sequences Encoding AOAH

A. Amino acid sequence of AOAH

AOAH was purified from DMSO-treated HL-60 cells and used to immunize mice. To induce antibodies, near-pure AOAH was adsorbed to lentil lectin-Sepharose (Pharmacia) and the resulting complex was injected intraperitoneally into mice. After fusion of mouse splenocytes with SP/2 cells, the resulting hybridomas were screened for the production of antibodies to AOAH using an activity depletion assay. One monoclonal antibody that depleted AOAH activity from solution also bound the 50 kD subunit of AOAH on Western blot analysis. The subunits of pure AOAH were then separated by reduction with 2-mercaptoethanol and separated on an SDS-PAGE gel, the 50 kD band was blotted onto a membrane, and the N-terminal amino acid sequence was determined by amino acid microsequence analysis. A 29 amino acid shown in Table 2 was designated the core sequence.

Table 2

30 AOAH Core Sequence

Xxx Asp Ile Xxx Ser Leu Pro Val Leu Ala Lys Ile
Xxx Gln Lys Ile Lys Leu Ala Met Glu Gln Xxx Val
Pro Phe Lys Asp Val

35

Two synthetic peptides were synthesized from a portion of the core sequence (Cys Ala Ala Ser Leu Pro Val Leu Ala Lys Ile Cys Gln Lys Leu Ala Met Glu Gln and Cys Ala Ala Ser Leu Pro Val Leu Ala Lys Ile Gly Gln Lys Leu Ala Met Glu Gln). Keyhole limpet hemocyanin was coupled to the peptide via the Cys residue of the Cys-Ala-Ala triplet of each peptide using the method essentially described by Green et al., Cell 28: 477-487, 1982). Sera from a peptide immunized rabbit also identified a 50 Kd protein by Western analysis.

B. Amplification of the Core Sequence

The DNA sequence encoding the disclosed amino acid sequence was isolated using the method essentially described by Lee et al. (Science 239: 1288-1291, 1988). Briefly, 1 µg of HL-60 poly(A)⁺ RNA, diluted into a total of 5 µl of 5 mM Tris pH 7.0, 0.05 mM EDTA, was heated at 65°C for 3 minutes, quick chilled in ice water, and reverse transcribed in 10 µl of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM MgCl₂, 500 µM dNTP, 0.5 µCi/µl α³²P-dATP containing 6 µg/µl random primer (Pharmacia LKB Biotechnology, Piscataway, NJ). The reaction was preincubated at 45°C for 5 minutes and was added to 20 U/µl MMLV(H-) reverse transcriptase (obtained from Bethesda Research Laboratories). Incubation was continued for one hour at 42°C. After incubation, TCA precipitable counts were determined. One microliter of the reaction mixture was added to 500 µl of water containing 100 µg of carrier RNA. The DNA was precipitated with 500 µl 20% TCA. One hundred microliters of this sample was counted directly to determine total counts in reaction. The remainder of the TCA sample was collected on a glass filter and washed with 10% TCA and counted. The synthesis yielded 250 ng of DNA.

Ninety microliters of 1 mM EDTA, 0.2 N KOH were added to the remainder of the reaction sample. The sample was incubated for 15 minutes at 65°C to hydrolyze the RNA.

The primer and small molecules were removed by an alkaline Sepharose 6B column chromatography, poured in a 1 ml disposable pipet, in 50 mM KOH, 0.1 mM EDTA. (The column was washed with 50 column volumes of buffer prior to use.)

- 5 The cDNA in the void volume was collected and ethanol precipitated after the addition of 5 μ g of carrier oyster glycogen. The random-primed cDNA was resuspended in 50 μ l of 10 mM Tris pH 7.4, 10 mM NaCl, and 0.1 mM EDTA.

- 10 Degenerate oligonucleotides were designed and synthesized to correspond to the terminal portions of the disclosed sequence and in addition contained sequences encoding terminal Eco RI sites to facilitate subcloning. The sense core primer family, ZC2388, (Table 3) and the antisense core primer family, ZC2295, (Table 3) were used
- 15 to amplify the cDNA using the method essentially described by Lee et al. (ibid.). Forty-two nanograms of random-primed cDNA was added to a reaction containing 1X PCR buffer (Perkin Elmer Cetus, Norwalk), 200 μ M dNTPs, 400 pmoles of ZC2388, 400 pmoles of ZC2295 and 2.5 U of Taq I
- 20 in a 100 μ l reaction. A mineral oil overlay was added to the reaction mixture, and the PCR reaction was carried out under the conditions shown in Table 4.

Table 3
Core Primer and Probe Families

	ZC2388 - Sense core primer family
5	23-mer, family of 256.
	Eco RI A A A
	CC C C A
	TCAGAATTCGTGTTGGCGAAGAT
	T T T
10	
	ZC2295 - Antisense core primer family
	26-mer, family of 128.
	Eco RI A A
	G T G C C
15	GATGAATTCACATCCTTAAAGGGGAC
	T T
	ZC2389 - 17-mer probe family of 128
	A A
20	GT C C A A
	AAACTGGCGATGGAGCAG
	T T
	ZC2298 - 26-mer I-probe family of 16
25	A A A A
	CAGAAGATIAAGITIGCIATGGAGCA

TABLE 4
Conditions for Polymerase Chain Reaction Amplification of
the AOAII Core Sequence

5	94°C for 3 minutes	
	30°C for 2 minutes	3 cycles
	72°C for 4 minutes	
	94°C for 2 minutes	
10	55°C for 2 minutes	40 cycles
	72°C for 4 minutes	

After the last cycle, the samples were extracted with 100 μ l chloroform to remove oil overlay. Five micrograms of oyster glycogen and 4 μ l 0.5 M EDTA were added, and the samples were phenol-chloroform extracted. The amplified DNA was ethanol precipitated and resuspended in 60 μ l of water.

The resuspended amplified DNA was cloned into the Eco RI site of a λ HG-3. To accomplish this, the amplified DNA was digested with Eco RI and the digest was run on a 4% NuSieve agarose TBE gel. Ultraviolet illumination revealed a faint band at about 71 nucleotides. This band was electrophoresed onto NA-45 paper (Schueller and Schneller) and was eluted by incubation at 65°C for 20 minutes in 400 μ l of 1.5 M NaCl, 10 mM Tris pH 7.4, and 0.1 mM EDTA. After the addition of 5 μ g of oyster glycogen, the sample was phenol-chloroform extracted three-times and ethanol precipitated. The DNA was resuspended in 10 μ l water.

The Eco RI-digested DNA was ligated with λ HG-3, which had been digested with Eco RI and dephosphorylated with calf alkaline phosphatase. The ligation mixture was incubated at room temperature for 2 hours. Gigapack Plus packaging mix (Stratagene Cloning Systems, Inc., La Jolla, CA) was added and the incubation was continued at room temperature for 2 hours. After incubation, 225 μ l of SM

buffer (Maniatis et al., *ibid.*) was added, and the solution was vortexed gently. Thirty microliters of chloroform was added, and the sample was gently vortexed. After a 2 minute centrifugation, the aqueous phase was
5 diluted 1/100 and 10 μ l was plated on *E. coli* Y1088 cells.

Plaque lifts were prepared using the method essentially described by Benton and Davis (*Science* 196: 180, 1977). Two oligonucleotide families of probes were designed that would identify the sequence between the core
10 PCR primers. The probe families, 2C2389 and 2C2298, consisted of a 17 mer of a family of 128 oligonucleotides and a 26 mer of a family of 16 oligonucleotides with inosines in the most ambiguous positions, respectively (Table 3). These oligonucleotides were kinased and
15 duplicate plaque lifts were probed with each probe family. Six positive plaques were chosen for further analysis.

Plate lysates were prepared from each isolate and lambda DNA was obtained by the method essentially described by Helms et al. (*DNA* 4: 39, 1985). Plasmids
20 residing in the λ vectors were released with the method called " λ -pop" essentially as described by Hagen (*ibid.*). Briefly, the λ isolates were digested with Not I, which liberates the complete pGEMT plasmid. The Not I-digested DNA was ligated for 5 hours at room temperature followed
25 by heat denaturation of the ligase at 65°C for 10 minutes. The ligation mixtures were phenol-chloroform extracted, ethanol precipitated, and the DNA was electroporated into *E. coli*. Plasmid DNA was prepared from the transformants using the method essentially described by Holmes and
30 Quigley (*Anal. Biochem.* 114: 193, 1981). The DNA sequence of the core fragment was determined by dideoxy-chain termination method on double stranded plasmid DNA, using a vector sequence primer. The translation of the DNA sequence showed that the deduced amino acid sequence
35 (Table 5) was identical to the core AOAH amino acid sequence and that it supplied the unknown amino acids at

positions 13 and 23 (Table 2) with Cys and Ser, respectively.

Table 5

5 AOAH Core Sequence Derived from PCR Amplification and
 Deduced Amino Acid Sequence

	Val	Leu	Ala	Lys	Ile	Cys	Gln	Lys	Ile	Lys	Leu	Ala	Met
5'	GTT	TTG	GCC	AAG	ATC	TGC	CAG	AAA	ATT	AAA	TTA	GCT	ATG
10	1		10		20		30						
	Glu	Gln	Ser	Val	Pro	Phe	Lys	Asp	Val				
	GAA	CAG	TCT	GTG	CCA	TTC	AAA	GAT	GT	3'			
	40		50		60								

15 C. Isolation of Partial cDNA Clones

Partial AOA cDNA clones were obtained from a cDNA library prepared from a DMSO-stimulated HL-60 cell RNA. RNA template for this library was prepared using the method essentially described by Chirgwin et al. (Biochemistry 18: 5294-5299, 1979). Poly(A)⁺ RNA was selected by two passages over an oligo-d(T) column. A lambda gt11 cDNA library was prepared from this RNA using an Invitrogen Inc. "Lambda Librarian" cDNA kit and a modification of the manufacturer's instructions. Briefly, double-stranded cDNA was synthesized using five micrograms of twice-selected poly(A)⁺ RNA and using the manufacturer's prescribed directions. The cDNA was blunted with T4 DNA polymerase, Eco RI adapters were added, and the cDNA was phosphorylated as described by the manufacturer. Following the phosphorylation, the cDNA was phenol-chloroform extracted two times. The cDNA was size-selected and the unincorporated primers were removed by chromatography on a 1.1 ml Sepharose 6B-CL column equilibrated with 10 mM Tris pH 7.4, 150 mM NaCl, and 0.1 mM EDTA. Fractions of the void volume containing the cDNA were pooled and the cDNA was ethanol precipitated. The cDNA was ligated to Eco RI-digested dephosphorylated

lambda gt11 (Clontech Inc.). The DNA was packaged using Gigapack Plus packaging mix (Stratagene Cloning Systems) and plated on *E. coli* Y1088 cells. A plate lysate library was prepared which contained 13.3 million independent isolates with a background of 1%. The actin positivity of the library was 0.34% (Hagen et al., *BioTechniques*, 6: 340, 1988). The plate lysate library was stored at 4°C over chloroform.

To screen the library for AOA^H cDNA clones, duplicate filter lifts were prepared from 20-150 mm plates containing 7.2 million phage. The filters were probed with radiolabeled ZC2465, which was designed from the core sequence. The probe was hybridized at 37°C in 20% Ullrich's hybridization buffer (Ullrich et al., *EMBO J.* 3: 361-364, 1984) and washed in 2X SSC (Maniatis et al, *ibid.*) at 50°C. Two potential positives, which appeared on duplicate lifts, were plaque purified and were designated clone 1.1 and clone 2.1. The cDNA inserts from these lambda clones were subcloned into p'EGT' and sequenced by double-stranded dideoxy-chain termination DNA sequence analysis. Sequence analysis of the cDNA inserts of clones 1.1 and 2.1 showed that the two clones were overlapping but incomplete and contained different sequences near the 5' ends and different coding capacities.

D. Preparation of 3' Template cDNA For Amplification of 3' AOA^H coding sequences

Complementary DNA was synthesized from U-937 poly (A)⁺ RNA utilizing ZC2487 (Table 1), which was designed to encode a oligo-d(T), Xho I, Sal I and Cla I primer. One microgram of U-937 poly(A)⁺-enriched RNA in 2 µl water was mixed with 2 µl 10 mM Tris, pH 7.0, 0.1 mM EDTA, heated at 65°C for 3 minutes and quick-chilled in ice water. Two microliters of 5x buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM MgCl₂) was mixed with 0.5 µl 10 mM dNTP, 1 µl of 5 pmole/µl ZC2487 for, 1.0 µl of

water, and 0.5 μ l 5 μ Ci/ μ l α^{32} P-dATP. The RNA solution was added to the reaction mixture and preincubated at 45°C for 5 minutes. One microliter of 200 unit/ μ l MMLV(H-) reverse transcriptase was added after the preincubation, and the sample was incubated for 1 hour at 45°C. After incubation, 1 μ l of 0.5 M EDTA and 1 μ l of 5 N KOH were added to the samples, and the RNA was hydrolyzed by incubation at 65°C for 15 minutes.

10 E. Preparation of 5' Template cDNA For Amplification of 5' AOA coding sequences

Complementary DNA was prepared to the 5' portion of the AOA coding sequence using essentially the method of Frohman et al. (ibid.). Complementary DNA was synthesized from U-937 poly(A)⁺ RNA utilizing the oligonucleotide primer ZC2487 encoding an antisense sequence corresponding to nucleotides 8 to 40 of the core sequence shown in Table 4. One microgram of poly(A)⁺-enriched RNA in 2 μ l of water was mixed with 2 μ l 10 mM Tris, pH 7.0 + 0.1 mM EDTA, heated at 65°C for 3 minutes and quick-chilled in ice water. Two microliters of 5x buffer was mixed with 0.5 μ l 10 mM dNTP, 1 μ l of 5 pmole/ μ l ZC2634, 1.0 μ l of water, and 0.5 μ l 5 μ Ci/ μ l α^{32} P-dATP. The RNA solution was added to the reaction mixture and preincubated at 45°C for 5 minutes. One microliter of 200 unit/ μ l MMLV(H-) reverse transcriptase was added after the preincubation, and the sample was incubated for one hour at 45°C. After incubation, 1 μ l of 0.25 M EDTA and 290 μ l of 0.05 N KOH were added to the samples, and the RNA was hydrolyzed by incubation at 65°C for 15 minutes.

The primers were removed from the cDNA sample by ultrafiltration through a Centricon Special YM-100 ultrafiltration unit in the presence of 50 mM KOH and 0.1 mM EDTA using conditions described by the manufacturer. After filtration the DNA was ethanol precipitated with the aid of 5 μ g of oyster glycogen. The DNA was resuspended in 5.7 μ l H₂O. The DNA was G-tailed by mixing the 5.7 μ l

of DNA with 0.3 μ l of water, 2 μ l of 5x TdT buffer (100 mM potassium cacodylate, pH 7.2, 2 mM CaCl_2 , 0.2 mM DTT, 1 mg/ml BSA) and 1 μ l of 1 mM dGTP. After a pre-incubation of 5 minutes at 15°C, 38.5 units of terminal
5 deoxynucleotidyl transferase (obtained from Collaborative Research) was added to the reaction mixture. The incubation was continued for an additional three minutes, and 90 μ l of 200 mM NaCl + 20 mM EDTA + 10 mM Tris pH 8.3 was added to stop the reaction. The DNA was ethanol
10 precipitated, washed with ethanol, and resuspended in 20 μ l of water.

For second strand synthesis of the tailed cDNA, 20 μ l of the G-tailed DNA was mixed with 47 μ l of water, 10 μ l of 10x PCR buffer(- MgCl_2) (500 mM NaCl, 100 mM Tris-Cl, pH 8.3 (at room temperature), 0.1 % gelatin. 16 μ l of
15 1.25 mM dNTPs, 4 μ l 50 mM MgCl_2 , 5 pmole of 202486, which encodes a poly d(C), Xho I, Sal I, Cla I primer. After the sample was preincubated at 94°C for 5 minutes, 5 units of Taq I were added followed by an oil overlay. The
20 incubation was continued at 40°C for 5 minutes, and 72°C for 15 minutes. One microliter of 250 mM EDTA was added to stop the reaction, and the sample was chloroform extracted to remove the oil overlay. Five micrograms of oyster glycogen was added and the sample was ethanol
25 precipitated.

F. Enrichment of 5' and 3' Template cDNAs

The 5' and 3' template cDNAs were fractionated by alkaline gel electrophoresis and the DNA from each gel
30 fraction was PCR amplified to identify the gel fragments containing amplifiable AOA coding sequences. An equal volume of 2x alkaline loading dye (60 mM NaOH, 4 mM EDTA, 20% glycerol, and 60% by volume bromocresol purple saturated with water) was added to the 3' template cDNA.
35 Half of the DNA in the 5' template cDNA was similarly prepared for electrophoresis and the DNA of the samples were fractionated on a 1% low melt alkaline agarose gel

(1% low-melt agarose in 30 mM NaOH + 2 mM EDTA). After electrophoresis, the agarose gel was cut into 12-0.5 cm fragments, representing DNA from 7,000 to 700 nucleotides for the 3' cDNA and in 8-1 cm fragments for the 5' cDNA.

5 The gel fragments were melted at 65°C. The melted gel fragments containing 3' template cDNA were diluted with 400 µl water for the 3' cDNA. The melted gel fragments containing 5' cDNA were used directly.

PCR amplification was performed with gel
10 fragments 1 to 12 for the 3' cDNA and gel fragments 2 to 6 for the 5' cDNA essentially as described by Frohman et al. (ibid.). Ten microliters each of the 3' template cDNA from fragments 1-12 was mixed with 10 µl 10x PCR Buffer(-MgCl₂), 57 µl of water, 16 µl of 1.23 mM dNTPs, 2 µl 50 mM
15 MgCl₂, 2 µl of 5 pmol/µl ZC2469 (encoding nucleotides 1 - 22 of the core sequence) and 2 µl of 5 pmol/µl ZC2489 (encoding the Xho I-Sal I-Cla I adaptor sequence). One microliter each of the 5' template cDNA from fragments 2-6 were mixed with 10 µl 10x PCR Buffer(-MgCl₂), 66 µl of
20 water, 16 µl of 1.25 mM dNTPs, 2 µl of 50 mM MgCl₂, 2 µl of 5 pmol/µl ZC2634 and 2 µl of 5 pmol/µl of ZC2633. Five units of Taq I DNA polymerase were added to each mixture during the first 94°C denaturation period, followed by a mineral oil overlay, and the mixtures were amplified
25 according to the conditions set forth in Table 6.

Table 6

Conditions for the Amplification of 3' DNA Sequences

TWO CYCLE:

30 94°C for 1 minute
50°C for 2 minutes
72°C for 3 minutes

Table 6 continued

FORTY CYCLES:

94°C for 1 minute

65°C for 2 minutes

5 72°C for 3 minutes

Conditions for the Amplification of 5' DNA Sequences

FORTY CYCLES:

10 94°C for 2 minutes

72°C for 4 minutes

ONE CYCLE:

72°C for 4 minutes

15

Following amplification, 10 µl each of the PCR reactions was electrophoresed on a 0.8% agarose gel after the addition of 2 µl of 5x loading dye (0.45 M Tris, 0.45 M boric acid, 0.01 M EDTA, 50% glycerol, 0.13% xylene cyanol, and 0.13% bromophenol blue). The gels were analyzed by visualization with ethidium bromide intercalation and UV-illumination followed by Southern blot analysis. Analysis of 3' PCR products showed a few minor bands by staining with ethidium bromide, but none of them proved to be the DNA band of interest as revealed by Southern blot analysis using a kinased antisense probe (ZC2470). The Southern blot hybridization pattern showed that the largest hybridizable band was at approximately 1500 nucleotides. This 1500 nucleotide band was most prominent from gel fragment #3, which contained template cDNA to 3000 nucleotides. The DNA eluted from fragment #3 was used in all additional experiments to obtain the 3' AOA coding sequences.

Analysis of the PCR products produced from the 5' PCR product did not reveal any DNA bands by ethidium bromide staining. Southern analysis with a random-primed 5' Eco RI-Xba I fragment of AOA cDNA clone 1.1 revealed a

35

hybridizable band at about 700 nucleotides from gel fragment #4.

Amplification of DNA from fragment #3 using ZC2469 and ZC2489 occasionally produced small quantities of the desirable DNA fragments; however smaller hybridizable bands were often the sole PCR product. Similarly, it was difficult to reproducibly obtain the desired 5' AOA PCR product. Finally, the PCR products would not clone via the restriction sites which were contained in the primers. Therefore the "Prime" sequence (described by Hagen in a co-pending commonly assigned US Patent Application Serial Number 07/320,191, which is incorporated by reference herein) was added to the primers and to facilitate the cloning of the PCR products after amplification.

G. Amplification of 5' and 3' AOA Coding Sequence

The DNA from fragment #3 was amplified using oligonucleotides ZC2631 and ZC2632. The 3' reaction mixture was prepared as follows. One microliter of DNA from fragment #3 was mixed with 10 μ l 10x PCR Buffer(-MgCl₂), 76 μ l of water, 16 μ l of 1.25 mM dNTPs, 2 μ l of 50 mM MgCl₂, 2 μ l each of 5 pmol/ μ l ZC2631 (the 5'prime-sense primer) and 5 pmol/ μ l ZC2632 encoding a prime sequence joined to the 3' end of the adapter sequence).

The DNA from fragment #4 was amplified using oligonucleotides ZC2633 and ZC2634. The 5' reaction mixture was prepared as follows. One microliter of gel fragment #4 was mixed with 10 μ l 10x PCR Buffer(-MgCl₂), 76 μ l of water, 16 μ l of 1.25 mM dNTPs, 2 μ l of 50 mM MgCl₂, 2 μ l each of 5 pmol/ μ l ZC2633 (the 5'prime-adapter primer) and 5 pmol/ μ l ZC2634 (the 3'prime-antisense primer). Five units of Taq I DNA polymerase and a mineral oil overlay were added to the sample during the 94°C denaturation step of the first cycle. The reaction mixtures were amplified according the conditions set forth in Table 7.

Table 7Conditions for Amplifying 5' and 3' Sequences Using Prime-primers

5

FORTY CYCLES:

94°C for 2 minutes

72°C for 4 minutes

10

ONE CYCLE:

72°C for 4 minutes

15

20

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30

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After amplification, 10 μ l of each sample was analyzed by agarose gel electrophoresis. Analysis of the gel revealed a minor band at 1500 nucleotides and major band at 800 for the 3' product. The amplification of the 5' DNA did not produce sufficient amounts of 5' PCR product after one round of RACE to clone the DNA. To produce enough 5' PCR product for subcloning, 10 μ l of DNA from the first amplification reaction was reamplified for 40 cycles, using the conditions set forth in Table 4. After amplification, all of the samples were chloroform, extracted followed by phenol/chloroform extraction. The DNAs were filtered on a Centricon Special YM100 microfiltration unit followed by ethanol precipitation with oyster glycogen as carrier. The PCR products were resuspended in water.

The PCR products were subcloned by treating the double-stranded DNA with T4 DNA polymerase in the presence of dATP to produce single-stranded prime sequences complementary to prime sequences present in the vector pVEGT'. The PCR product were each mixed with 1 μ l of 10x T4 buffer, 1 μ l of 1 mM dATP, 1 μ l of 50 mM DTT and 1 unit of T4 DNA polymerase. The reactions were incubated 1 hour at 15°C. The polymerase was heat denatured at 65°C for 15 minutes.

The T4 DNA polymerase cut-back PCR products were electrophoresed in a 0.8% low melt agarose gel, and the 1500 bp and 800 bp bands were gel-purified. The cut-back DNA was ligated with the cut-back pVEGT'. Plasmid DNA was prepared from the transformants using the method essentially described by Holmes and Quigley (ibid.). Analysis of the plasmid DNA by Eco RI restriction analysis revealed 6 clones containing the 1500 bp 3' cDNA and 5 clones containing the 800 bp 5' cDNA. DNA sequence analysis of these clones confirmed that the clones contained DNA sequences that overlapped the core sequence and comparison with the DNA sequence of the 1.1 and 1.2 cDNA clones established a full length sequence of 2290 bp.

15 H. Amplification of Full Length AOA H cDNA

Full length cDNAs were generated by PCR amplification from the DNA from fragment #3 using oligonucleotide primers designed from sequences from the 5' and 3' clones. Oligonucleotides ZC2703, encoding the prime sequence joined to the 5' end of the sequence of nucleotides 38 to 61 of Figure 1, and ZC2704, encoding the prime sequence joined to the 3' end of an antisense sequence corresponding to nucleotides 2175 to 2198 of Figure 1, were used in two separate series of PCR reactions to obtain two independently derived AOA H clones. In one reaction, 1 μ l of fragment #3 was amplified in a 100 μ l volume PCR reaction (1X Taq 1 DNA polymerase buffer (Promega Biotech), 200 μ M dNTP, 0.25 μ M of each primer, 22.5 units/ml of Promega Biotech Taq 1 DNA polymerase) under the conditions set forth in Table 8. Ten microliters of the above-described reaction was subjected to a second round of amplification under conditions identical to those described above. The resulting PCR product was designated as C. In a second reaction, 10 μ l of fragment #3 was used as the starting template for 2 rounds of 30 cycles of PCR amplification identical to those described above except that the Promega Biotech Taq

1 DNA polymerase was used at a concentration of 45 units/ml. The resulting PCR product was designated as 4.

Table 8

Conditions for Amplifying Full-length AOAH cDNA

5

THIRTY CYCLES:

94°C for 2 minutes

72°C for 4 minutes

10

ONE CYCLE:

72°C for 4 minutes

cool to room temperature

15

After the two rounds of PCR as described above, samples C and 4 were extracted with chloroform to remove the oil overlay. One hundred microliters of 20 mM Tris pH 8.3 + 200 mM NaCl + 20 mM EDTA was added, and the solution was extracted with phenol-chloroform. The primers were separated from the PCR products by ultrafiltration with Amicon Centricon Special YM100 ultrafiltration device as described by the manufacture using water as the eluate. Five micrograms of oyster glycogen was added and the DNA was ethanol precipitated. The PCR products and the Eco RI-linearized pDVEG (Example 1C) were exonucleased using T4 DNA polymerase to expose the prime sequences. As described in Example 2G, the PCR products were treated in a 10 µl T4 DNA polymerase reaction. After 1 hour, the samples were incubated for 15 minutes at 65 C to inactivate the polymerase. The DNA was electrophoresed on a 0.8% low melt agarose gel, and the amplified DNA was gel-purified. The cut-back PCR products were each ligated to the cut-back pDVEG.

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The DNA was electroporated into *E. coli* DH5αFTM (Bethesda Research Labs) and plated. Plasmid DNA was prepared, and the DNA analyzed by restriction endonuclease analysis. One clone from C and one clone from 4 were chosen and designated C/26 and 4-33. Clones C/26 and 4-33

were sequenced by the dideoxy chain-termination method. The sequences of C/26 and 4-33 are shown in Figures 1 and 2, respectively.

5 I. Construction of a Consensus DNA Sequence.

Because PCR synthesis can incorporate mutations into amplified DNA, depending on the number of cycles and conditions of DNA synthesis, it was necessary to be rigorous about establishing a consensus sequence. By
10 comparing the sequences of the two full length PCR clones, the 5' AOAHP PCR products, two 3' AOAHP products, and the two partial cDNA clones, a consensus AOAHP cDNA sequence was established as shown in Figure 3. A comparison of the
15 sequences showed that clone 4-33 (Figure 2) had three PCR-induced mutations resulting in three amino acid changes and clone C/26 (Figure 1) had nine PCR-induced mutations resulting in seven amino acid changes.

A consensus DNA sequence encoding AOAHP was constructed from the AOAHP clone 4-33 (Figure 2) which has
20 the fewest (three) PCR-induced mutations: A to G at position 601, changing Serine 117 to Asparagine; A to G at position 1444, changing Arginine 398 to Glutamine; T to C at position 1505, changing Serine 418 to Leucine. These lesions are corrected as follows to construct a consensus
25 DNA sequence termed AOAHP1 (Figure 4).

Standard protocols for PCR template preparation and PCR reaction conditions were followed (Innis, Gelfand, Sninsky, and White, T.J., eds. PCR Protocols, Academic Press, 1990). Specifically, the PCR template was a 2.2 kb
30 Eco RI fragment of clone Zem229R-4-33 (Example 3). As shown in Figure 10, oligonucleotides ZC3074, a sense primer which inserts a mammalian consensus ribosome binding site (encoding the nucleotide sequence CCACC) just
upstream of the initiation Methionine, preceded by Sal I
35 and Eco RI sites and encodes nucleotides 252 to 269 of Figure 2, and ZC3075, an antisense primer which corresponds to nucleotides 576 to 596 and inserts an Xho I

site at position 584, were synthesized and used to amplify 4-33 DNA. After amplification, the DNA was digested with Eco RI and Xho I to isolate a 346 bp Eco RI-Xho I fragment encoding nucleotides 2-348 of Figure 4.

5 The mutations at positions 601 and 1444 of clone 4-33 (Figure 2) were corrected by PCR amplification using oligonucleotides ZC3076, a sense oligonucleotide primer corresponding to nucleotides 576 to 607 of Figure 1 which creates an Xho I site by making a silent mutation at
10 position 584 and which corrects the mutation at position 601, and ZC3077, an antisense oligonucleotide primer corresponding to nucleotides of Figure 1 which creates a Bam HI site at position 1481 by making silent mutations at
15 positions 1481, 1482 and 1483 and corrects the mutation at position 1444. As shown in Figure 10, the 1.2 kb Eco RI fragment of 4-33 DNA was subjected to PCR amplification using oligonucleotides ZC3076 and 3077. The amplified DNA was digested with Xho I and Bam HI to isolate an 897 bp Xho I-Bam HI fragment encoding nucleotides 348 to 1245 of
20 Figure 4.

 The mutation at position 1505 of clone 4-33 was corrected by PCR amplification. As shown in Figure 10, oligonucleotides ZC3078, a sense primer corresponding to nucleotides 1473 to 1511 of Figure 2 which inserts the
25 same Bam HI site as ZC3077 and corrects the mutation at position 1505 of Figure 2, and ZC3079, an antisense primer which corresponds to nucleotides 1967 to 1999 of Figure 2 and which inserts Sal I and Eco RI sites immediately downstream of the stop codon were used to amplify 4-33
30 DNA. The amplified DNA was digested with Bam HI and Sal I to isolate a 507 nucleotide Bam HI-Sal I fragment encoding nucleotides 1246 to 1752 of Figure 4.

 The PCR products were subcloned into pUC18 as shown in Figure 10. The Eco RI-Xho I fragment, comprising
35 the 5' sequence of AOAII encoding nucleotides 2 to 348 of Figure 4, and the Xho I-Bam HI fragment, comprising an AOAII sequence corresponding to nucleotides 348 to 1245 of

Figure 4 having the corrected sequence at positions 367 and 1210 (corresponding to positions 602 and 1444 of Figure 1, respectively), were ligated together with Eco RI-Bam HI-digested pUC18. The resulting plasmids were confirmed by sequence analysis. A clone having the correct insert was digested with Eco RI and Bam HI to isolate the Eco RI-Bam HI fragment, comprising the AOA sequence corresponding to positions 2 to 1245 of Figure 4. In a separate reaction, the Bam HI-Sal I fragment, comprising an AOA sequence corresponding to nucleotides 1246 to 1752 of Figure 4 having the corrected sequence at position 1270 (corresponding to position 1504 of Figure 1) was ligated with Bam HI-Sal I-digested pUC18.

After the AOA-specific portions of the above ligation were confirmed by sequence analysis, the partial AOA cDNA were reassembled in a mammalian expression vector as shown in Figure 10. The Bam HI-Eco RI fragment, comprising an AOA sequence corresponding to nucleotides 1246 to 1752 of Figure 4, was isolated from a plasmid clone having the correct insert. The Eco RI-Bam HI fragment and the Bam HI-Eco RI fragment were joined with Eco RI-linearized Zem229R by ligation. The resulting plasmid containing the AOA1 sequence from Figure 4 was designated PRS431 (Figure 10).

25

Example 3

Expression of AOA in Cultured Mammalian Cells

A. Construction of Mammalian Expression Vectors Zem229R-C/26 and Zem229R-4-33

The AOA cDNAs contained in plasmids C/26 and 4-33 were subcloned into the mammalian expression vector Zem229R. Plasmids C/26 and 4-33 were digested with Eco RI to isolate the AOA cDNA. Plasmid Zem229R was linearized by digestion with Eco RI and treated with calf alkaline phosphatase to prevent recircularization. The cDNAs from C/26 and 4-33 were each ligated with the linearized Zem229R. Plasmid clones having the C/26 and 4-33 inserts

in the correct orientation relative to the promoter were designated Zem229R-C/26 and Zem229R-4-33, respectively. Plasmid Zem229R-C/26 and Zem229R-4-33 have been deposited as *E. coli* transformants with the American Type Culture Collection (Rockville, MD).

B. Construction of Acyloxyacyl Hydrolase DNA Sequences Containing A Synthetic Signal Sequence

The partial 5' consensus DNA sequence subcloned into pUC18 as described in Example 2.I. was further modified to replace a portion of the native pre sequence with the amino acid sequence Pro-Gly-Ala-Trp-Ala (referred to hereinafter as PGAWA), which was designed using the rules set forth by von Heinje (ibid.). In addition, the synthetic leader was constructed both in the presence and the absence of the pro sequence. The partial 5' consensus AOA DNA sequence present as an Eco RI-Bam HI insert in pUC18 described in Example 2.I. was digested with Eco RI to isolate the approximately 1.25 kb fragment. A portion of the 2.2 kb Eco RI fragment was amplified using the oligonucleotide primers ZC3202 and ZC3203 (Table 9). Approximately 50 to 100 μ g of the Eco RI fragment in 2 μ l of water was mixed with 50 pmoles of each primer, 8 μ l of a solution containing 1.25 mM of each nucleotide triphosphate, 5 μ l of 10x Cetus Buffer and 2.5 units of Taq I polymerase. The reaction mixture was brought to a final volume of 50 μ l with distilled water. The reaction mixture was subjected to two cycles of one minute at 94°C, one minute at 40°C and two minutes at 72°C followed by twenty cycles of fifteen seconds at 94°C, fifteen seconds at 55°C and two minutes at 72°C. The final cycle was followed by a seven minute incubation at 72°C. The PCR product was gel purified and digested with Spe I and Bgl II. The resulting 365 bp fragment was gel purified.

56

Table 9

ZC3202

TTA ATT TTC TGG CAG ATC TTG GCC

5

ZC3203

TAG GGT GTG TAC TAG TGG TGT CTG

ZC3112

10

CGA ATT CCA CCA TGC AGT CCC CCT GGA AAA TCC TTA
CGG TGG TGC CTC TAT TCT TGC TCC TGT CTC CA

ZC3113

15

GGC GCC TGG GCT TCT CCA GCC AAC GAT GAC CAG TCC
AGG CCC AGC CTC TCG AAT GGG CAC ACC TGT GTA GGG
TGT GTA

ZC3114

20

CTA GTA CAC ACC CTA CAC AGG TGT GCC CAT TCG AGA
GGC TGG GCC TGG ACT GGT CAT CGT TGG CTG GAG AAG
CCC A

ZC3115

25

GGC GCC TGG AGA CAG GAG CAA GAA TAG AGG CAC CAC
CGT AAG GAT TTT CCA GGG GGA CTG CAT GGT GGA ATT
CGA GCT

ZC3116

30

CTA GAA GTG GTA GAA GAA AGA GAA GCG ACA TTT GTT
CAC TCC CGG TTT TGG CCA A

ZC3117

35

GAT CTT GGC CAA AAC CGG GAG TGA ACA AAT GTC GCA
TCT CTT TCT TCT ACC ACT T

ZC3118

CTA GTA CAC ACC CTA CAC AGG TGT GCC CAT TCG AGA
GAG CCC A

Table 9 continued

ZC3119

GGC GCC TGG GCT CTC TCG AAT GGG CAC ACC TGT GTA
GGG TGT GTA

5

Oligonucleotide adapters were designed to encode a pre sequence having PGAWA sequence with the pro sequence. Oligonucleotides ZC3112, ZC3113, ZC3114 and ZC3115 (Table 9) were designed to encode, when annealed, an Sst I-Spe I adapter encoding PGAWA sequence with the pro sequence. Oligonucleotides ZC3113 and ZC3115 (Table 9) were kinased using the method essentially described by Sambrook et al. (Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989; which is incorporated herein by reference). The kinase was heat inactivated, and the kinased oligonucleotides were mixed with oligonucleotides ZC3112 and ZC3114 (Table 9). The Oligonucleotide mixture was annealed by heating the reaction to 65°C and allowing the mixture to cool to room temperature for one hour. The 152 bp Sst I-Spe I adapter was purified by polyacrylamide gel electrophoresis. Oligonucleotides ZC3112, ZC3119, ZC3115 and ZC3118 (Table 9) were designed to encode, when annealed, an Sst I-Spe I adapter encoding PGAWA sequence without the pro sequence. Oligonucleotides ZC3119 and ZC3115 (Table 9) were kinased using the method essentially described by Sambrook et al. (ibid.). The kinase was heat inactivated, and the kinased oligonucleotides were mixed with oligonucleotides ZC3112 and ZC3118 (Table 9). The oligonucleotide mixture was annealed by heating the reaction to 65°C and allowing the mixture to cool to room temperature for one hour. The 119 bp Sst I-Spe I adapter was purified by polyacrylamide gel electrophoresis.

The partial 5' consensus ADAM DNA sequence, which was subcloned as an Eco RI-Bam HI into pUC18 (Example 2.I.), was isolated as an approximately 1250 bp Eco RI-Bam HI fragment and was subcloned into Eco RI-Bam

HI digested pIC19H. The resultant plasmid was linearized by digestion with Bgl II and Sst I. The linearized Bgl II-Sst I fragment was ligated with the Spe I-Bgl II PCR-generated fragment and with either the

5 ZC3112:ZC3119/ZC3115:ZC3118 adapter encoding the PGAWA without the pro sequence or the
ZC3112:ZC3113/ZC3115:ZC3114 adapter encoding the PGAWA with the pro sequence to generate plasmids which were designated pCC-4-pro #4 and pCC-3+pro #1, respectively.
10 Plasmids pCC-4-pro #4 and pCC-3+pro #1 were digested with Eco RI and Bam HI to isolate the approximately 1.25 kb fragments. The 3' consensus AOA H DNA sequence was obtained as an Bam HI-Eco RI fragment from the Bam HI-Sal I subclone described in Example 2.I and shown in Figure
15 10. The Eco RI-Bam HI fragments from pCC-4-pro #4 and pCC-3+pro #1 were each subcloned with the Bam HI-Eco RI fragment encoding the 3' consensus AOA H sequence into pZem229R which had been linearized by digestion with Eco RI. A plasmid comprising the complete AOA H cDNA sequence
20 containing the pre sequence containing PGAWA without the pro sequence in pZem229R was designated pRS433, and a plasmid comprising the complete AOA H cDNA sequence containing the pre sequence containing PGAWA with the pro sequence in pZem229R was designated pRS432.

25

C. Construction of Acyloxyacyl Hydrolase DNA Sequences
Containing A Proteolytic Cleavage Sequence Between
The Small and Large Subunit Sequences of AOA H

30

The cDNA sequences present in plasmids pCC-4-pro #4 and pCC-3+pro #1 were each modified to insert a
proteolytic cleavage sequence between the small and large
subunit sequences. Plasmids pCC-4-pro #4 and pCC-3+pro #1
were each digested with Eco RI and Bgl II to isolate the
35 vector-containing fragment. Plasmid pCC-4-pro #4 was digested with Eco RI and Xba I to isolate the 433 bp fragment, and pCC-3+pro #1 was digested with Eco RI and

Xba I to isolate the 466 bp. Oligonucleotides ZC3116 and ZC3117 (Table 9), which were designed and synthesized to form an Xba I-Bgl II adapter encoding the proteolytic cleavage signal Arg-Arg-Lys-Arg (Sequence I.D. No. 12) were kinase and annealed using methods essentially described by Sambrook et al. (ibid.). The Eco RI-Bgl II fragment of pCC-4-pro #4, the 433 bp Eco RI-Xba I fragment of pCC-4-pro #4 and the ZC3116/ZC3117 adapter (Table 9) were ligated. A resulting plasmid was designated PGAWA-Pro + RRKR. The Eco RI-Bgl II fragment of pCC-3+pro #1, the 466 bp Eco RI-Xba I fragment of pCC-3-pro #1 and the ZC3116/ZC3117 adapter were ligated. A resulting plasmid was designated PGAWA+Pro + RRKR. Plasmids PGAWA-Pro + RRKR and PGAWA+Pro + RRKR were each digested with Eco RI and Bam HI to isolate the AOA coding sequences. The 3' consensus AOA DNA sequence was obtained as a Bam HI-Eco RI fragment from the Bam HI-Sal I subclone described in Example 2.I and shown in Figure 10. The Eco RI-Bam HI fragment from PGAWA-Pro + RRKR and PGAWA+Pro - RRKR were each ligated with the Bam HI-Eco RI fragment encoding the 3' consensus AOA DNA sequence and pZem229R which had been linearized by digestion with Eco RI. A plasmid comprising the AOA coding sequence with the synthetic pre sequence, the pro region and the proteolytic cleavage sequence was designated pRS434. A plasmid comprising the AOA coding sequence with the synthetic pre sequence and the proteolytic cleavage sequence but lacking the pro sequence was designated pRS435.

30 D. Expression of AOA cDNAs in Mammalian Cells

Plasmids Zem229R-C/26 and Zem229R-4-33 were transfected into BHK 570 cells (deposited with the American Type Culture Collection under accession number 10314) using the calcium phosphate-mediated transfection method essentially described by Chen et al. (BioTechniques 6: 632:, 1988). After the transfected cells were grown for three days, 10 ml of media was taken for each

transfection and from a negative control and stored at 4°C. The transfections and a negative control were trypsinized and resuspended in 10 ml of media and counted. The cells were centrifuged and the media was discarded.

5 The cell pellet was resuspended in 1x PBS (phosphate-buffered saline, Sigma Chemical Co., St. Louis, MO) + 0.1% Triton X-100 + 1 mM PMSF to a concentration of 1×10^6 cells/100 μ l. The resuspended cells were lysed for 10 minutes at room temperature and the lysates were
10 centrifuged at 10,000 rpm in a microfuge for 10 minutes at 4°C. The supernatants were removed and placed on ice.

The duplicate spent media samples and duplicate lysate supernatants were assayed for AOAH activity using the method essentially described by Munford and Hall
15 (Science 234: 203-205, 1986). Briefly, 100 μ l of media or lysate was added to 400 μ l of reaction buffer, pH 4.8 (1.25 mg/ml BSA, 0.625% Triton X-100, 187.5 mM NaCl, 6.25 mM $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 25 mM Tris-Base, 12.5 mM citric acid) containing 1 μ l $^{14}\text{C}/^3\text{H}$ -LPS Substrate. The reaction
20 mixtures were incubated for approximately 16 hours at 37°C. After incubation, 1 ml of cold 100% ethanol was added and the mixtures were vortexed and chilled on ice for 45 minutes. The samples were centrifuged for 10 minutes at 4°C at 10,000 rpm. One milliliter of
25 supernatant from each sample was removed and lyophilized in a glass scintillation vial. Five milliliters of Optifluor scintillation fluid (Packard Instrument Co, Downers Grove, IL) was added and the samples were counted for 2 minutes on the tritium channel. The results of the
30 assay are shown in Table 10.

Table 10
AOAH Activity Assay Results

	<u>Sample</u>	<u>Counts</u>
	Positive Control:	
5	1 μ l 1:100 dilution of purified AOAH	2799
		2984
	Negative Control:	
	reaction buffer	184
		153
10	Negative Control:	
	BHK 570 cell media	241
		175
	Negative Control:	
15	BHK 570 cells	184
		181
	Zem229R-C/26 media	193
		539
20	Zem229R-C/26 cells	450
		477
	Zem229R-4-33 media	193
		214
25	Zem229R-4-33 cells	2866
		3091

30 As shown in Table 9, the Zem229R-C/26 and Zem229R-4-33 transfectants contained AOAH activity with Zem229R-C/26 transformants having only approximately 18% of the activity of the Zem229R-4-33 transfectants.

35 Plasmids Zem229R-4-33 and pRS431 were each transfected into BHK 570 cells by calcium phosphate precipitation as described above. Transfectants were lysed and the lysates were assayed for activity

essentially as described above. As shown in Table 11, both the Zem229R-4-33 and the pRS431 transfectants had AOA^H activity with pRS431 transfectants, which express the consensus AOA^H1 sequence of Figure 4, having more than
5 twice the activity as the Zem229R-4-33 transfectants.

Table 11

AOA^H Activity Assay Results

10	Sample	<u>H³-fatty acid released, dpm/mg cell protein</u>
	Zem229R-4-33 cells	2038
	pRS431 cells	4973

The deacylation of LPS using the recombinant
15 AOA^H purified from Zem229R-4-33 transfectants was measured as the maximal percent of tritiated fatty acids released from AOA^H-treated, labeled LPS using a protocol essentially as described in U.S. Patent 4,929,604, which is incorporated herein by reference.

20 Biosynthetically labeled LPS was deacylated with enzyme contained in an extract of Zem299-4-33 transfected cells. An extract of cells that did not contain the transfected DNA was incubated with the LPS under identical conditions as a control. After incubation, the reaction
25 mixtures were extracted with chloroform/methanol and the chloroform extracts (which contained the ³H-fatty acids) were counted. Calculation of the percent of the ³H radioactivity that was released from the LPS by the extract of Zem99-4-33 transfected cells indicated that
30 approximately 27% of the fatty acids were removed; the extract of untransfected cells removed less than 2%. This level of deacylation is consistent with the fact that only one third of the fatty acyl chains in LPS are susceptible to cleavage by AOA^H. In addition, in the same
35 experiment a large excess of purified neutrophil AOA^H released 27% of the tritiated fatty acids from the LPS.

Plasmid pRS431-transfected cells were step-wise amplified in medium containing first 10 μ M methotrexate and finally 25 μ M methotrexate. The AOA_H produced from mammalian cells transfected with pRS431 was activated in vitro using trypsin. AOA_H produced from pRS431-transfected cells grown in serum free medium (Table 12) for five days was activated by the addition of 10 μ l of 20 μ g/ml trypsin (Sigma, St. Louis, MO) and 80 μ l of buffer (1x PBS (Sigma, St. Louis, MO) + 0.1 % Triton X-100) to ten microliter aliquots of the spent serum free medium. The reaction mixtures were incubated at 30°C for 20 minutes. After incubation, 400 μ l of activity assay mixture (Table 10) and 2 μ g of ³H-LPS was added to each sample. The reaction mixtures were incubated at 37°C for one hour. After incubation, the reaction mixtures were precipitated with the addition of 1 ml of cold ethanol. The precipitates were removed by centrifugation and the supernatants were counted. The results showed trypsin-activated AOA_H had 10- to 30-fold more activity than untreated AOA_H.

Table 12

<u>Serum Free Medium</u>		
	5 ml	29.23 mg/ml L-glutamine
25	5 ml	100 mM NaPyruvate
	12.5 ml	1 M Hepes
	500 μ l	10 mg/ml insulin
	1 ml	5 mg/ml fetuin
	5000 μ l	10 mg/ml transferrin
30	375 μ l	4 μ g/l selenium
	5 ml	100x PSN (GIBCO-BRL, Gaithersburg, MD)
	500 μ l	1 mM methotrexate

Add the ingredients to 500 ml Dulbecco's Minimal Eagles Medium. Store at 4°C.

Table 12 continued200 mM Tris-Citrate buffer

2.42 g Tris base
5 2.1 g citrate

Dissolve the solids in distilled water and bring to a final volume of 100m.

10 Activity Assay Buffer

500 mg BSA (Sigma, St. Louis, MO)
2.5 ml Triton X-100
4.5 g NaCl
15 0.367 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

Dissolve in 200 mM Tris Citrate buffer and bring to a final volume of 400 ml.

Plasmids pRS432, pRS433, pRS434 and pRS435 were
20 each transfected into BHK 570 cells as described previously. The selected transfectants and their spent media were assayed for AOAH activity using the method described above, and each transfectant was shown to produce active AOAH.

25 From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the
30 invention is not limited except as by the appended claims.

WHAT IS CLAIMED IS:

1. An isolated DNA sequence encoding acyloxyacyl hydrolase wherein said acyloxyacyl hydrolase comprises a small subunit and a large subunit.
2. An isolated DNA sequence according to claim 1 wherein said DNA sequence is a cDNA sequence
3. A DNA sequence according to claim 1 wherein said DNA sequence comprises the DNA sequence of Figure 1 from nucleotide 354 to nucleotide 1976, the DNA sequence of Figure 2 from nucleotide 354 to nucleotide 1976, the DNA sequence of Figure 3 from nucleotide 389 to nucleotide 2011 or the DNA sequence of Figure 4 from nucleotide 120 to nucleotide 1742.
4. A DNA sequence according to claim 1 wherein said acyloxyacyl hydrolase comprises the amino acid sequence of Figure 1 from Leucine, number 35 to Histidine, number 575; the amino acid sequence of Figure 2 from Leucine, number 35 to Histidine, number 575; the amino acid sequence of Figure 3 from Leucine, number 35 to Histidine, number 575 or the amino acid sequence of Figure 4 from Leucine, number 35 to Histidine, number 575.
5. A DNA sequence according to claim 1 wherein said DNA sequence further encodes between the small and large subunits the amino acid sequence $(R_1)_n-R_2-R_3$, wherein R_1 , R_2 and R_3 are Lys or Arg and $n = 0, 1, 2, 3, \text{ or } 4$.
6. An isolated DNA sequence encoding the small subunit of acyloxyacyl hydrolase.
7. An isolated DNA sequence according to claim 6 wherein said DNA sequence is a cDNA sequence
8. A DNA sequence according to claim 6 wherein said DNA sequence comprises a DNA sequence of Figure 1 from

nucleotide 354 to nucleotide 713, the DNA sequence of Figure 2 from nucleotide 354 to nucleotide 713, the DNA sequence of Figure 3 from nucleotide 389 to nucleotide 748 or the DNA sequence of Figure 4 from nucleotide 120 to nucleotide 479.

9. A DNA sequence according to claim 6 wherein said small subunit of acyloxyacyl hydrolase comprises the amino acid sequence of Figure 1 from Leucine, number 35, to Arginine, number 154; the amino acid sequence of Figure 2 from Leucine, number 35, to Arginine, number 154; the amino acid sequence of Figure 3 from Leucine, number 35, to Arginine, number 154 or the amino acid sequence of Figure 4 from Leucine, number 35, to Arginine, number 154.

10. An isolated DNA sequence encoding the large subunit of acyloxyacyl hydrolase.

11. An isolated DNA sequence according to claim 10 wherein said DNA sequence is a cDNA sequence

12. A DNA sequence according to claim 10 wherein said DNA sequence comprises a DNA sequence encoding the DNA sequence of Figure 1 from nucleotide 720 to nucleotide 1976, the DNA sequence of Figure 2 from nucleotide 720 to nucleotide 1976, the DNA sequence of Figure 3 from nucleotide 755 to nucleotide 2011 or the DNA sequence of Figure 4 from nucleotide 486 to nucleotide 1742.

13. A DNA sequence according to claim 10 wherein said large subunit of acyloxyacyl hydrolase comprises the amino acid sequence of Figure 1 from Serine, number 157, to Histidine, number 575; the amino acid sequence of Figure 2 from Serine, number 157, to Histidine number 575; the amino acid sequence of Figure 3 from Serine, number 157, to Histidine, number 575 or the amino acid sequence of Figure 4 from Serine, number 157, to Histidine, number 575.

14. A DNA construct comprising the following operably linked elements:

a transcriptional promoter;

a DNA sequence encoding acyloxyacyl hydrolase

wherein said DNA sequence encodes a small subunit and a large subunit; and

a transcriptional terminator.

15. A DNA construct according to claim 14 wherein said DNA construct further comprises at least one secretory signal sequence operably linked to said DNA sequence encoding acyloxyacyl hydrolase.

16. A DNA construct according to claim 15 wherein said secretory signal sequence comprises the acyloxyacyl hydrolase secretory signal sequence, the tissue plasminogen activator secretory signal sequence, the α -2 plasmin inhibitor secretory signal sequence, the Saccharomyces cerevisiae BAR1 secretory signal sequence or the Saccharomyces cerevisiae MF α 1 secretory signal sequence.

17. A DNA construct according to claim 16 wherein said DNA construct further comprises a DNA sequence encoding the C-terminal domain of the Saccharomyces cerevisiae BAR1 gene operably linked to said DNA sequence.

18. A DNA construct according to claim 15 wherein said secretory signal sequence encodes the amino acid sequence Met-Glu-Ser-Pro-Trp-Lys-Ile-Leu-Thr-Val-Ala-Pro-Leu-Phe-Leu-Leu-Leu-Ser-Pro-Gly-Ala-Trp-Ala-Ser-Pro-Ala-Asn-Asp-Asp-Gln-Ser-Arg-Pro-Ser or Met-Glu-Ser-Pro-Trp-Lys-Ile-Leu-Thr-Val-Ala-Pro-Leu-Phe-Leu-Leu-Leu-Ser-Pro-Gly-Ala-Trp-Ala.

19. A DNA construct according to claim 14 wherein said DNA sequence further encodes between said small and large subunits the amino acid sequence $(R_1)_n-R_2-R_3$, wherein R_1 , R_2 and R_3 are Lys or Arg and $n = 0, 1, 2$ or 3 .

20. A DNA construct according to claim 14 wherein said DNA sequence comprises the DNA sequence of Figure 1 from nucleotide 354 to nucleotide 1976, the DNA sequence of Figure 2 from nucleotide 354 to nucleotide 1976, the DNA sequence of Figure 3 from nucleotide 389 to nucleotide 2011 or the DNA sequence of Figure 4 from nucleotide 120 to nucleotide 1742.

21. A DNA construct according to claim 14 wherein said acyloxyacyl hydrolase comprises the amino acid sequence of Figure 1 from Leucine, number 35 to Histidine, number 575; the amino acid sequence of Figure 2 from Leucine, number 35 to Histidine, number 575; the amino acid sequence of Figure 3 from Leucine, number 35 to Histidine, number 575 or the amino acid sequence of Figure 4 from Leucine, number 35 to Histidine, number 575.

22. A DNA construct comprising the following operably linked elements:

- a transcriptional promoter;
- a DNA sequence encoding the small subunit of acyloxyacyl hydrolase; and
- a transcriptional terminator.

23. A DNA construct according to claim 22 wherein said DNA construct further comprises at least one secretory signal sequence operably linked to said DNA sequence encoding the small subunit of acyloxyacyl hydrolase.

24. A DNA construct according to claim 23 wherein said secretory signal sequence comprises the acyloxyacyl hydrolase secretory signal sequence, the tissue plasminogen activator secretory signal sequence, the α -2 plasmin inhibitor secretory signal sequence, the Saccharomyces cerevisiae BAR1 secretory signal sequence or the Saccharomyces cerevisiae MFol secretory signal sequence.

25. A DNA construct according to claim 24 wherein said DNA construct further comprises a DNA sequence encoding the C-terminal domain of the Saccharomyces cerevisiae BAR1 gene operably linked to said DNA sequence.

26. A DNA construct according to claim 23 wherein said secretory signal sequence encodes the amino acid sequence Met-Glu-Ser-Pro-Trp-Lys-Ile-Leu-Thr-Val-Ala-Pro-Leu-Phe-Leu-Leu-Leu-Ser-Pro-Gly-Ala-Trp-Ala-Ser-Pro-Ala-Asn-Asp-Asp-Gln-Ser-Arg-Pro-Ser or Met-Glu-Ser-Pro-Trp-Lys-Ile-Leu-Thr-Val-Ala-Pro-Leu-Phe-Leu-Leu-Leu-Ser-Pro-Gly-Ala-Trp-Ala.

27. A DNA construct according to claim 22 wherein said DNA sequence comprises the DNA sequence of Figure 1 from nucleotide 354 to nucleotide 713, the DNA sequence of Figure 2 from nucleotide 354 to nucleotide 713, the DNA sequence of Figure 3 from nucleotide 389 to nucleotide 743 or the DNA sequence of Figure 4 from nucleotide 120 to nucleotide 479.

28. A DNA construct according to claim 22 wherein said small subunit of acyloxyacyl hydrolase comprises the amino acid sequence of Figure 1 from Leucine, number 35, to Arginine, number 154; the amino acid sequence of Figure 2 from Leucine, number 35, to Arginine number 154; the amino acid sequence of Figure 3 from Leucine, number 35, to Arginine, number 154 or the amino acid sequence of Figure 4 from Leucine, number 35, to Arginine, number 154.

29. A DNA construct comprising the following operably linked elements:
a transcriptional promoter;
a DNA sequence encoding the large subunit of acyloxyacyl hydrolase; and
a transcriptional terminator.

30. A DNA construct according to claim 29 wherein said DNA construct further comprises at least one secretory

signal sequence operably linked to said DNA sequence encoding the large subunit of acyloxyacyl hydrolase.

31. A DNA construct according to claim 30 wherein said secretory signal sequence comprises the acyloxyacyl hydrolase secretory signal sequence, the tissue plasminogen activator secretory signal sequence, the α -2 plasmin inhibitor secretory signal sequence, the Saccharomyces cerevisiae BAR1 secretory signal sequence or the Saccharomyces cerevisiae MF α 1 secretory signal sequence.

32. A DNA construct according to claim 31 wherein said DNA construct further comprises a DNA sequence encoding the C-terminal domain of the Saccharomyces cerevisiae BAR1 gene operably linked to said DNA sequence.

33. A DNA construct according to claim 30 wherein said secretory signal sequence encodes the amino acid sequence Met-Glu-Ser-Pro-Trp-Lys-Ile-Leu-Thr-Val-Ala-Pro-Leu-Phe-Leu-Leu-Leu-Ser-Pro-Gly-Ala-Trp-Ala-Ser-Pro-Ala-Asn-Asp-Asp-Gln-Ser-Arg-Pro-Ser or Met-Glu-Ser-Pro-Trp-Lys-Ile-Leu-Thr-Val-Ala-Pro-Leu-Phe-Leu-Leu-Leu-Ser-Pro-Gly-Ala-Trp-Ala.

34. A DNA construct according to claim 29 wherein said DNA sequence comprises the DNA sequence of Figure 1 from nucleotide 720 to nucleotide 1976, the DNA sequence of Figure 2 from nucleotide 720 to nucleotide 1976, the DNA sequence of Figure 3 from nucleotide 755 to nucleotide 2011 or the DNA sequence of Figure 4 from nucleotide 486 to nucleotide 1742.

35. A DNA construct according to claim 29 wherein said large subunit of acyloxyacyl hydrolase comprises the amino acid sequence of Figure 1 from Serine, number 157, to Histidine, number 575; the amino acid sequence of Figure 2 from Serine, number 157, to Histidine number 575; the amino acid sequence of Figure 3 from Serine, number 157, to

Histidine, number 575 or the amino acid sequence of Figure 4 from Serine, number 157, to Histidine, number 575.

36. A cultured eukaryotic host cell containing a DNA construct comprising the following operably linked elements:

- a transcriptional promoter;
- an isolated DNA sequence encoding acyloxyacyl hydrolase wherein said DNA sequence encodes a small subunit and a large subunit; and
- a transcriptional terminator.

37. A eukaryotic cell according to claim 36 wherein the DNA construct further comprises a secretory signal sequence operably linked to said DNA sequence encoding acyloxyacyl hydrolase.

38. A eukaryotic cell according to claim 37 wherein said secretory signal sequence comprises the acyloxyacyl hydrolase secretory signal sequence, the tissue plasminogen activator secretory signal sequence, the α -2 plasmin inhibitor secretory signal sequence, the Saccharomyces cerevisiae BAR1 secretory signal sequence or the Saccharomyces cerevisiae MF α 1 secretory signal sequence.

39. A eukaryotic cell according to claim 38 wherein said DNA construct further comprises a DNA sequence encoding the C-terminal domain of the Saccharomyces cerevisiae BAR1 gene operably linked to said DNA sequence.

40. A eukaryotic cell according to claim 36 wherein said secretory signal sequence encodes the amino acid sequence Met-Glu-Ser-Pro-Trp-Lys-Ile-Leu-Thr-Val-Ala-Pro-Leu-Phe-Leu-Leu-Leu-Ser-Pro-Gly-Ala-Trp-Ala-Ser-Pro-Ala-Asn-Asp-Asp-Gln-Ser-Arg-Pro-Ser or Met-Glu-Ser-Pro-Trp-Lys-Ile-Leu-Thr-Val-Ala-Pro-Leu-Phe-Leu-Leu-Leu-Ser-Pro-Gly-Ala-Trp-Ala.

41. A eukaryotic cell according to claim 36 wherein said DNA sequence comprises the DNA sequence of Figure 1 from nucleotide 354 to nucleotide 1976, the DNA sequence of Figure 2 from nucleotide 354 to nucleotide 1976, the DNA sequence of Figure 3 from nucleotide 389 to nucleotide 2011 or the DNA sequence of Figure 4 from nucleotide 120 to nucleotide 1742.

42. A eukaryotic cell according to claim 36 wherein said acyloxyacyl hydrolase comprises the amino acid sequence of Figure 1 from Leucine, number 35, to Histidine, number 575; the amino acid sequence of Figure 2 from Leucine, number 35, to Histidine, number 575; the amino acid sequence of Figure 3 from Leucine, number 35, to Histidine, number 575 or the amino acid sequence of Figure 4 from Leucine, number 35, to Histidine, number 575.

43. A eukaryotic cell according to claim 36 wherein said cell is a cultured mammalian cell or a yeast cell.

44. A eukaryotic cell according to claim 36 wherein said DNA sequence further encodes the amino acid sequence $(R_1)_n-R_2-R_3$, wherein R_1 , R_2 and R_3 are Lys or Arg and $n = 0, 1, 2$ or 3 , between said small and large subunits.

45. A eukaryotic host cell transformed or transfected with a first DNA construct containing the information necessary to direct the expression of the large subunit of acyloxyacyl hydrolase and a second DNA construct containing the information necessary to direct the expression of the small subunit of acyloxyacyl hydrolase.

46. A eukaryotic cell according to claim 45 wherein said cell is a cultured mammalian cell or a yeast cell.

47. A eukaryotic cell containing a first DNA construct containing the information necessary to direct the secretion of the large subunit of acyloxyacyl hydrolase and a

second DNA construct containing the information necessary to direct the secretion of the small subunit of acyloxyacyl hydrolase.

48. A eukaryotic cell according to claim 47 wherein said cell is a cultured mammalian cell or a yeast cell.

49. A method for producing acyloxyacyl hydrolase comprising the steps of:

(a) growing cultured eukaryotic cells transformed or transfected with a DNA construct containing the information necessary to direct the expression of acyloxyacyl hydrolase; and

(b) isolating the acyloxyacyl hydrolase from said cells.

50. A method according to claim 49 wherein said eukaryotic cells are cultured mammalian cells or yeast cells.

51. A method for producing acyloxyacyl hydrolase comprising the steps of:

(a) growing eukaryotic cells transformed or transfected with a DNA construct containing the information necessary to direct the secretion of acyloxyacyl hydrolase; and

(b) isolating the acyloxyacyl hydrolase from said cells.

52. A method according to claim 51 wherein said eukaryotic cells are cultured mammalian cells or yeast cells.

53. A method for producing acyloxyacyl hydrolase comprising the steps of:

(a) growing eukaryotic cells transformed or transfected with a first DNA construct containing the information necessary to direct the expression of the large subunit of acyloxyacyl hydrolase and a second DNA construct

containing the information necessary to direct the expression of the small subunit of acyloxyacyl hydrolase; and

(b) isolating the acyloxyacyl hydrolase from the cells.

54. A method according to claim 53 wherein said eukaryotic cells are cultured mammalian cells or yeast cells.

55. A method for producing acyloxyacyl hydrolase comprising the steps of:

(a) growing eukaryotic cells transformed or transfected with a first DNA construct containing the information necessary to direct the secretion of the large subunit of acyloxyacyl hydrolase and a second DNA construct containing the information necessary to direct the secretion of the small subunit of acyloxyacyl hydrolase; and

(b) isolating the acyloxyacyl hydrolase from the cells.

56. A method according to claim 55 wherein said eukaryotic cells are cultured mammalian cells or yeast cells.

57. A method for treating a mammal with a gram-negative bacterial sepsis comprising administering to said animal a therapeutically effective amount of recombinant acyloxyacyl hydrolase.

10 20 30 40 50 60
 CCTCCAGCTC TTTGTGTGTG GCTCTCTCAG GGTCCAACAA GAGCAAGCTG TGGGTCTGTG
 70 80 90 100 110 120
 AGTGTATTATG TGTGCTTTTA TTCAC TTCAC ACTTATTGAA AAGTGTGTAT GTGAGAGGGT
 130 140 150 160 170 180
 GGGGTGTGTG TGTCAAAGAG AGTGAGGAAG AGAAGGAGAG AGAGATCAAT TGATTCTGCA
 190 200 210 220 230 240
 GCCTCAGCTC CAGCATCCCT CAGTTGGGAG CTTCCAAAGC CGGGTGATCA CTTGGGGTGC
 250 260 269 278 287
 ATAGCTCGGA G ATG CAG TCC CCC TGG AAA ATC CTT ACG GTG GCG CCT CTA
 Met Gln Ser Pro Trp Lys Ile Leu Thr Val Ala Pro Leu
 296 305 314 323 332
 TCC TTG CTC CTG TCT CTT CAG TCC TTG GCC TCT CCA GCC AAC GAT GAC
 Ser Leu Leu Leu Ser Leu Gln Ser Leu Ala Ser Pro Ala Asn Asp Asp
 341 350 359 368 377 386
 CAG TCC AGG CCC AGC CTC TCG AAT GGG CAC ACC TGT GTA GGG TGT GTG
 Gln Ser Arg Pro Ser Leu Ser Asn Gly His Thr Cys Val Gly Cys Val
 395 404 413 422 431
 CTG GTG GTG TCT GTA ATA GAA CAG CTT GCT CAA GTT CAC AAC TCG ACG
 Leu Val Val Ser Val Ile Glu Gln Leu Ala Gln Val His Asn Ser Thr
 440 449 458 467 476
 GTC CAG GCC TCG ATG GAG AGA CTG TGC AGC TAC CTG CCT GAA AAA CTG
 Val Gln Ala Ser Met Glu Arg Leu Cys Ser Tyr Leu Pro Glu Lys Leu
 485 494 503 512 521 530
 TTC TTG AAA ACC ACC TGC TAT TTA GTC ATT GAC AAG TTT GGA TCA GAC
 Phe Leu Lys Thr Thr Cys Tyr Leu Val Ile Asp Lys Phe Gly Ser Asp
 539 548 557 566 575
 ATC ATA AAA CTG CTT AGC GCA GAT ATG AAT GCT GAT GTG GTA TGT CAC
 Ile Ile Lys Leu Leu Ser Ala Asp Met Asn Ala Asp Val Val Cys His
 584 593 602 611 620
 ACT CTG GAG TTT TGT AGA CAG AAC ACT GGC CAA CCA TTG TGT CAT CTC
 Thr Leu Glu Phe Cys Arg Gln Asn Thr Gly Gln Pro Leu Cys His Leu
 629 638 647 656 665 674
 TAC CCT CTT CCC AAG GAG ACA TGG AAA TTT ACA CTA CAG AAG GCA AGA
 Tyr Pro Leu Pro Lys Glu Thr Trp Lys Phe Thr Leu Gln Lys Ala Arg
 683 692 701 710 719
 CAA ATT ATC AAG AAG TCC CCG ATT CTG AAA TAT TCT AGA AGT GGT TCT
 Gln Ile Ile Lys Lys Ser Pro Ile Leu Lys Tyr Ser Arg Ser Gly Ser
 728 737 746 755 764
 GAC ATT TGT TCA CTC CCG GTT TTG GCC AAG ATC TGC CAG AAA ATT AAA
 Asp Ile Cys Ser Leu Pro Val Leu Ala Lys Ile Cys Gln Lys Ile Lys

FIG. 1A.

SUBSTITUTE SHEET

773	782	791	800	809	818
TTA GCT ATG	GAA CAG TCT	GTG CCA TTC	AAA GAT GTG	GAT TCA GAC	AAA
Leu Ala Met	Glu Gln Ser	Val Pro Phe	Lys Asp Val	Asp Ser Asp	Lys
827	836	845	854	863	
TAC AGC GTT	CTC CCA ACA	CTG CGG GGC	TAT CAC TGG	CGG GGG	AGA GAC
Tyr Ser Val	Leu Pro Thr	Leu Arg Gly	Tyr His Trp	Arg Gly Arg	Asp
872	881	890	899	908	
TGT AAT GAC	AGC GAC GAG	TCA GTG TAC	CCA GGT AGA	AGG CCG	AAC AAC
Cys Asn Asp	Ser Asp Glu	Ser Val Tyr	Pro Gly Arg	Arg Pro Asn	Asn
917	926	935	944	953	962
TGG GAT GTC	CAT CAG GAT	TCA AAC TGT	AAT GGC ATT	TGG GGT GTC	GAT
Trp Asp Val	His Gln Asp	Ser Asn Cys	Asn Gly Ile	Trp Gly Val	Asp
971	980	989	998	1007	
CCA AAA GAT	GGA GTT CCA	TAT GAG AAG	AAA TTC TGT	GAA GGT TCA	CAG
Pro Lys Asp	Gly Val Pro	Tyr Glu Lys	Lys Phe Cys	Glu Gly Ser	Gln
1016	1025	1034	1043	1052	
CCC AGG GGA	ATC ATT TTG	CTG GGA GAC	TCA GCT GGG	GCT CAT TTT	CAC
Pro Arg Gly	Ile Ile Leu	Leu Gly Asp	Ser Ala Gly	Ala His Phe	His
1064	1070	1079	1088	1097	1106
ATC TCT CCT	GAA TGG ATC	ACA GCG TCG	CAG ATG TCT	TTG AAC TCT	TTT
Ile Ser Pro	Glu Trp Ile	Thr Ala Ser	Gln Met Ser	Leu Asn Ser	Phe
1115	1124	1133	1142	1151	
ATC AAT CTA	CCA ACA GCC	CTT ACC AAC	GAG CTT GAC	TGG CCC CAA	CTC
Ile Asn Leu	Pro Thr Ala	Leu Thr Asn	Glu Leu Asp	Trp Pro Gln	Leu
1160	1169	1178	1187	1196	
TCT GGT GCT	ACA GGA TTT	CTG GAC TCC	ACT GTT GGA	ATT AAA GAA	AAA
Ser Gly Ala	Thr Gly Phe	Leu Asp Ser	Thr Val Gly	Ile Lys Glu	Lys
1206	1214	1223	1232	1241	1250
TCT ATT TAC	CTT CGC TTA	TGG AAA AGA	AAC CAC TGT	AAT CAC AGG	GAC
Ser Ile Tyr	Leu Arg Leu	Trp Lys Arg	Asn His Cys	Asn His Arg	Asp
1259	1268	1277	1286	1295	
TAC CAG AAT	ATT TCA AGA	AAT GGT GCA	TCT TCC CGA	AAC CTG AAG	AAA
Tyr Gln Asn	Ile Ser Arg	Asn Gly Ala	Ser Ser Arg	Asn Leu Lys	Lys
1304	1313	1322	1331	1340	
TTT ATA GAA	AGC TTG TCT	AGA AGC AAG	GTG TTG GAC	TAT CCC GCC	ATC
Phe Ile Glu	Ser Leu Ser	Arg Ser Lys	Val Leu Asp	Tyr Pro Ala	Ile
1350	1358	1367	1376	1385	1394
GTT ATA TAT	GCC ATG ATT	GGA AAT GAT	GTC TGC AGT	GGG AAG AGT	GAC
Val Ile Tyr	Ala Met Ile	Gly Asn Asp	Val Cys Ser	Gly Lys Ser	Asp
1403	1412	1421	1430	1439	
CCA GTC CCA	GCC ATG ACC	ACT CCT GAG	AAA CTC TAC	TCC AAC GTC	ATG
Pro Val Pro	Ala Met Thr	Thr Pro Glu	Lys Leu Tyr	Ser Asn Val	Met

FIG. 1B.**SUBSTITUTE SHEET**

1448 CAG ACT CTG AAG CAT CTA AAT TCC CAC CTG CCC AAT GGC AGC CAT GTT
 Gln Thr Leu Lys His Leu Asn Ser His Leu Pro Asn Gly Ser His Val
 1494 ATT TTG TAT GGC TTA CCA 1511 GGA ACC 1520 TTT CTC TGG 1529 GAT AAT TTG 1538 CAC
 Ile Leu Tyr Gly Leu Pro Asp Gly Thr Phe Leu Trp Asp Asn Leu His
 1547 AAC AGA TAT CAT CCT CTC GGC CAG CTA AAT AAA GAC ATG ACC 1593 TAT GGC
 Asn Arg Tyr His Pro Leu Gly Gln Leu Asn Lys Asp Met Thr Tyr Ala
 1592 CAG TTG TAC TCC 1601 TTC CTG AAC 1610 TGC TTC CAG 1619 GTC AGC CCC 1628 TGC CAC GGC
 Gln Leu Tyr Ser Phe Leu Asn Cys Phe Gln Val Ser Pro Cys His Gly
 1638 TGG ATG TCT TCC AAC AAG ACG TTG CGG ACT CTC ACT TCA GAG AGA 1682 GCA
 Trp Met Ser Ser Asn Lys Thr Leu Arg Thr Leu Thr Ser Glu Arg Ala
 1691 GAG CAA CTC TCC AAC 1700 ACA CTG AAA 1709 AAA ATT GCA 1718 GCC AGT GAG 1727 AAA TTT
 Glu Gln Leu Ser Asn Thr Leu Lys Lys Ile Ala Ala Ser Glu Lys Phe
 1736 ACA AAC TTC AAT CTT TTC TAC ATG GAT TTT GCC TTC CAT GAA ATC ATA
 Thr Asn Phe Asn Leu Phe Tyr Met Asp Phe Ala Phe His Glu Ile Ile
 1782 CAG GAG TGG 1790 CAG AAG AGA 1799 GGC GGA CAG 1808 CCC TGG CAG 1817 CTC ATC GAG 1826 CCC
 Gln Glu Trp Gln Lys Arg Gly Gly Gln Pro Trp Gln Leu Ile Glu Pro
 1835 GTG GAT GGA TTC CAC CCC AAC GAG GTG GCT TTG CTG TTG TTG GCG GAT
 Val Asp Gly Phe His Pro Asn Glu Val Ala Leu Leu Leu Leu Ala Asp
 1880 CAT TTC TGG AAA 1889 AAG GTG CAG 1898 CTC CAG TGG 1907 CCC CAA ATC CTG GGA AAG
 His Phe Trp Lys Lys Val Gln Leu Gln Trp Pro Gln Ile Leu Gly Lys
 1926 GAG AAT CCG TTC AAC CCC CAG ATT AAA CAG GTG TTT GGA GAC CAA GGC
 Glu Asn Pro Phe Asn Pro Gln Ile Lys Gln Val Phe Gly Asp Gln Gly
 1979 GGG CAC TGAGCCTCTC 1989 AGGAGCATGC 1999 ACCCCTGGGG 2009 AGCACAGGGA 2019 GGCAGAGGCT
 Gly His
 2020 TGGGTAAACT 2039 CATTCCACAA 2049 ACCCTATGGG 2059 GGCTGCCACG 2069 TCACAGGCC 2079 AAAGGACTCT
 2090 TCTTCAGCAG 2099 CATCTTTGCA 2109 AAATGTCTTT 2119 CTCTCAATGA 2129 AGAGCATATC 2139 TGGACGACTG
 2150 TGCAATGCTG 2159 TGTGCTC

FIG. 1C.
SUBSTITUTE SHEET

10 20 30 40 50 60
 CCTCCAGCTC TTTGTGTGTG GCTCTCTCAG GGTCCAACAA GAGCAAGCTG TGGGTCTGTG
 70 80 90 100 110 120
 AGTGTTTATG TGTGCTTTTA TTCAC TTCAC ACTTATTGAA AAGTGTGTAT GTGAGAGGGT
 130 140 150 160 170 180
 GGGGTGTGTG TGTCAAAGAG AGTGAGGAAG AGAAGGAGAG AGAGATCAAT TGATTCTGCA
 190 200 210 220 230 240
 GCCTCAGCTC CAGCATCCCT CAGTTGGGAG CTTCCAAAGC CGGGTGATCA CTTGGGGTGC
 250 260 269 278 287
 ATAGCTCGGA G ATG CAG TCC CCC TGG AAA ATC CTT ACG GTG GCG CCT CTA
 Met Gln Ser Pro Trp Lys Ile Leu Thr Val Ala Pro Leu
 296 305 314 323 332
 TTC TTG CTC CTG TCT CTT CAG TCC TCG GCC TCT CCA GCC AAC GAT GAC
 Phe Leu Leu Leu Ser Leu Gln Ser Ser Ala Ser Pro Ala Asn Asp Asp
 341 350 359 368 377 386
 CAG TCC AGG CCC AGC CTC TCG AAT GGG CAC ACC TGT GTA GGG TGT GTG
 Gln Ser Arg Pro Ser Leu Ser Asn Gly His Thr Cys Val Gly Cys Val
 395 404 413 422 431
 CTG GTG GTG TCT GTA ATA GAA CAG CTT GCT CAA GTT CAC AAC TCG ACG
 Leu Val Val Ser Val Ile Glu Gln Leu Ala Gln Val His Asn Ser Thr
 440 449 458 467 476
 GTC CAG GCC TCG ATG GAG AGA CTG TGC AGC TAC CTG CCT GAA AAA CTG
 Val Gln Ala Ser Met Glu Arg Leu Cys Ser Tyr Leu Pro Glu Lys Leu
 485 494 503 512 521 530
 TTC TTG AAA ACC ACC TGC TAT TTA GTC ATT GAC AAG TTT GGA TCA GAC
 Phe Leu Lys Thr Thr Cys Tyr Leu Val Ile Asp Lys Phe Gly Ser Asp
 539 548 557 566 575
 ATC ATA AAA CTG CTT AGC GCA GAT ATG AAT GCT GAT GTG GTA TGT CAC
 Ile Ile Lys Leu Leu Ser Ala Asp Met Asn Ala Asp Val Val Cys His
 584 593 602 611 620
 ACT CTG GAG TTT TGT AAA CAG AGC ACT GGC CAA CCA TTG TGT CAT CTC
 Thr Leu Glu Phe Cys Lys Gln Ser Thr Gly Gln Pro Leu Cys His Leu
 629 638 647 656 665 674
 TAC CCT CTT CCC AAG GAG ACA TGG AAA TTT ACA CTA CAG AAG GCA AGA
 Tyr Pro Leu Pro Lys Glu Thr Trp Lys Phe Thr Leu Gln Lys Ala Arg
 683 692 701 710 719
 CAA ATT GTC AAG AAG TCC CCG ATT CTG AAA TAT TCT AGA AGT GGT TCT
 Gln Ile Val Lys Lys Ser Pro Ile Leu Lys Tyr Ser Arg Ser Gly Ser
 728 737 746 755 764
 GAC ATT TGT TCA CTC CCG GTT TTG GCC AAG ATC TGC CAG AAA ATT AAA
 Asp Ile Cys Ser Leu Pro Val Leu Ala Lys Ile Cys Gln Lys Ile Lys

FIG. 2A.

SUBSTITUTE SHEET

773 TTA GCT ATG Leu Ala Met	782 GAA CAG TCT Glu Gln Ser	791 GTG CCA TTC Val Pro Phe	800 AAA GAT GTG Lys Asp Val	809 GAT TCA GAC Asp Ser Asp	818 AAA Lys
TAC AGC GTT TTC CCA ACA CTG CGG GGC TAT CAC TGG CGG GGG AGA GAC Tyr Ser Val Phe Pro Thr Leu Arg Gly Tyr His Trp Arg Gly Arg Asp	827 GTT TTC CCA ACA CTG CGG GGC TAT CAC TGG CGG GGG AGA GAC Val Phe Pro Thr Leu Arg Gly Tyr His Trp Arg Gly Arg Asp	836 ACA CTG CGG GGC TAT CAC TGG CGG GGG AGA GAC Thr Leu Arg Gly Tyr His Trp Arg Gly Arg Asp	845 GGC TAT CAC TGG CGG GGG AGA GAC Gly Tyr His Trp Arg Gly Arg Asp	854 TGG CGG GGG AGA GAC Trp Arg Gly Arg Asp	863 AGA GAC Arg Asp
872 AAT GAC AGC GAC GAG TCA GTG TAC CCA GGT AGA AGG CCG AAC AAC Cys Asn Asp Ser Asp Glu Ser Val Tyr Pro Gly Arg Arg Pro Asn Asn	881 GAC GAG TCA GTG TAC CCA GGT AGA AGG CCG AAC AAC Asp Glu Ser Val Tyr Pro Gly Arg Arg Pro Asn Asn	890 GTG TAC CCA GGT AGA AGG CCG AAC AAC Val Tyr Pro Gly Arg Arg Pro Asn Asn	899 GGT AGA AGG CCG AAC AAC Gly Arg Arg Pro Asn Asn	908 CCG AAC AAC Pro Asn Asn	
917 TGG GAT GTC CAT CAG GAT TCA AAC TGT AAT GGC ATT TGG GGT GTC GAT Trp Asp Val His Gln Asp Ser Asn Cys Asn Gly Ile Trp Gly Val Asp	926 CAT CAG GAT TCA AAC TGT AAT GGC ATT TGG GGT GTC GAT His Gln Asp Ser Asn Cys Asn Gly Ile Trp Gly Val Asp	935 TCA AAC TGT AAT GGC ATT TGG GGT GTC GAT Ser Asn Cys Asn Gly Ile Trp Gly Val Asp	944 AAT GGC ATT TGG GGT GTC GAT Asn Gly Ile Trp Gly Val Asp	953 TGG GGT GTC GAT Trp Gly Val Asp	962 GAT Asp
971 AAA GAT GGA GTT CCA TAT GAG AAG AAA TTC TGT GAA GGT TCA CAG Pro Lys Asp Gly Val Pro Tyr Glu Lys Lys Phe Cys Glu Gly Ser Gln	980 GAT GGA GTT CCA TAT GAG AAG AAA TTC TGT GAA GGT TCA CAG Asp Gly Val Pro Tyr Glu Lys Lys Phe Cys Glu Gly Ser Gln	989 AAG AAA TTC TGT GAA GGT TCA CAG Lys Lys Phe Cys Glu Gly Ser Gln	998 TGT GAA GGT TCA CAG Cys Glu Gly Ser Gln	1007 TCA CAG Ser Gln	
1016 AGG GGA ATC ATT TTG CTG GGA GAC TCA GCT GGG GCT CAT TTT CAC Pro Arg Gly Ile Ile Leu Leu Gly Asp Ser Ala Gly Ala His Phe His	1025 ATT TTG CTG GGA GAC TCA GCT GGG GCT CAT TTT CAC Ile Ile Leu Leu Gly Asp Ser Ala Gly Ala His Phe His	1034 GGA GAC TCA GCT GGG GCT CAT TTT CAC Gly Asp Ser Ala Gly Ala His Phe His	1043 GCT GGG GCT CAT TTT CAC Ala Gly Ala His Phe His	1052 CAT TTT CAC His Phe His	
1064 TCT CCT GAA TGG ATC ACA GCG TCG CAG ATG TCT TTG AAC TCT TTC Ile Ser Pro Glu Trp Ile Thr Ala Ser Gln Met Ser Leu Asn Ser Phe	1070 CCT GAA TGG ATC ACA GCG TCG CAG ATG TCT TTG AAC TCT TTC Pro Glu Trp Ile Thr Ala Ser Gln Met Ser Leu Asn Ser Phe	1079 ATC ACA GCG TCG CAG ATG TCT TTG AAC TCT TTC Thr Ala Ser Gln Met Ser Leu Asn Ser Phe	1088 CAG ATG TCT TTG AAC TCT TTC Gln Met Ser Leu Asn Ser Phe	1097 TCT TTG AAC TCT TTC Ser Leu Asn Ser Phe	1106 TCT TTC Ser Phe
1115 AAT CTA CCA ACA GCC CTT ACC AAC GAG CTT GAC TGG CCC CAA CTC Ile Asn Leu Pro Thr Ala Leu Thr Asn Glu Leu Asp Trp Pro Gln Leu	1124 CCA ACA GCC CTT ACC AAC GAG CTT GAC TGG CCC CAA CTC Pro Thr Ala Leu Thr Asn Glu Leu Asp Trp Pro Gln Leu	1133 GCC CTT ACC AAC GAG CTT GAC TGG CCC CAA CTC Thr Ala Leu Thr Asn Glu Leu Asp Trp Pro Gln Leu	1142 CTT GAC TGG CCC CAA CTC Leu Asp Trp Pro Gln Leu	1151 CAA CTC Gln Leu	
1160 GGT GCT ACA GGA TTT CTG GAC TCC ACT GTT GGA ATT AAA GAA AAA Ser Gly Ala Thr Gly Phe Leu Asp Ser Thr Val Gly Ile Lys Glu Lys	1169 GCT ACA GGA TTT CTG GAC TCC ACT GTT GGA ATT AAA GAA AAA Gly Ala Thr Gly Phe Leu Asp Ser Thr Val Gly Ile Lys Glu Lys	1178 GGA TTT CTG GAC TCC ACT GTT GGA ATT AAA GAA AAA Thr Gly Phe Leu Asp Ser Thr Val Gly Ile Lys Glu Lys	1187 TGT GGA ATT AAA GAA AAA Val Gly Ile Lys Glu Lys	1196 AAA GAA AAA Lys Glu Lys	
1206 ATT TAC CTT CGC TTA TGG AAA AGA AAC CAC TGT AAT CAC AGG GAC Ser Ile Tyr Leu Arg Leu Trp Lys Arg Asn His Cys Asn His Arg Asp	1214 TAC CTT CGC TTA TGG AAA AGA AAC CAC TGT AAT CAC AGG GAC Tyr Leu Arg Leu Trp Lys Arg Asn His Cys Asn His Arg Asp	1223 CTT CGC TTA TGG AAA AGA AAC CAC TGT AAT CAC AGG GAC Leu Arg Leu Trp Lys Arg Asn His Cys Asn His Arg Asp	1232 TGT AAT CAC AGG GAC Cys Asn His Arg Asp	1241 AAT CAC AGG GAC Asn His Arg Asp	1250 GAC Asp
1259 CAG AAT ATT TCA AGA AAT GGT GCA TCT TCC CGA AAC CTG AAG AAA Tyr Gln Asn Ile Ser Arg Asn Gly Ala Ser Ser Arg Asn Leu Lys Lys	1268 AAT ATT TCA AGA AAT GGT GCA TCT TCC CGA AAC CTG AAG AAA Asn Ile Ser Arg Asn Gly Ala Ser Ser Arg Asn Leu Lys Lys	1277 GAT GCA TCT TCC CGA AAC CTG AAG AAA Asp Val Ser Ser Arg Asn Leu Lys Lys	1286 TCT TCC CGA AAC CTG AAG AAA Ser Ser Arg Asn Leu Lys Lys	1295 AAG AAA Lys Lys	
1304 ATA GAA AGC TTG TCT AGA AAC AAG GTG TTG GAC TAT CCC GCC ATC Phe Ile Glu Ser Leu Ser Arg Asn Lys Val Leu Asp Tyr Pro Ala Ile	1313 GAA AGC TTG TCT AGA AAC AAG GTG TTG GAC TAT CCC GCC ATC Ile Glu Ser Leu Ser Arg Asn Lys Val Leu Asp Tyr Pro Ala Ile	1322 TTG TCT AGA AAC AAG GTG TTG GAC TAT CCC GCC ATC Ser Arg Asn Lys Val Leu Asp Tyr Pro Ala Ile	1331 GTG TTG GAC TAT CCC GCC ATC Val Leu Asp Tyr Pro Ala Ile	1340 TAT CCC GCC ATC Tyr Pro Ala Ile	
1350 ATA TAT GCC ATG ATT GGA AAT GAT GTC TGC AGT GGG AAG AGT GAC Val Ile Tyr Ala Met Ile Gly Asn Asp Val Cys Ser Gly Lys Ser Asp	1358 TAT GCC ATG ATT GGA AAT GAT GTC TGC AGT GGG AAG AGT GAC Tyr Ala Met Ile Gly Asn Asp Val Cys Ser Gly Lys Ser Asp	1367 GCC ATG ATT GGA AAT GAT GTC TGC AGT GGG AAG AGT GAC Ala Met Ile Gly Asn Asp Val Cys Ser Gly Lys Ser Asp	1376 AAT GAT GTC TGC AGT GGG AAG AGT GAC Asn Asp Val Cys Ser Gly Lys Ser Asp	1385 AGT GGG AAG AGT GAC Ser Gly Lys Ser Asp	1394 AGT GAC Ser Asp
1403 GTC CCA GCC ATG ACC ACT CCT GAG AAA CTC TAC TCC AAC GTC ATG Pro Val Pro Ala Met Thr Thr Pro Glu Lys Leu Tyr Ser Asn Val Met	1412 CCA GCC ATG ACC ACT CCT GAG AAA CTC TAC TCC AAC GTC ATG Val Pro Ala Met Thr Thr Pro Glu Lys Leu Tyr Ser Asn Val Met	1421 GTC CCA GCC ATG ACC ACT CCT GAG AAA CTC TAC TCC AAC GTC ATG Pro Glu Lys Leu Tyr Ser Asn Val Met	1430 TAC TCC AAC GTC ATG Tyr Ser Asn Val Met	1439 GTC ATG Val Met	

FIG. 2B.

SUBSTITUTE SHEET

1448 1457 1466 1475 1484
 CGG ACT CTG AAG CAT CTA AAT TCC CAC CTG CCC AAT GGC AGC CAT GTT
 Arg Thr Leu Lys His Leu Asn Ser His Leu Pro Asn Gly Ser His Val
 1494 1502 1511 1520 1529 1538
 ATT TTG TAT GGC TCA CCA GAT GGA ACC TTT CTC TGG GAT AAT TTG CAC
 Ile Leu Tyr Gly Ser Pro Asp Gly Thr Phe Leu Trp Asp Asn Leu His
 1547 1556 1565 1574 1593
 AAC AGA TAT CAT CCT CTC GGC CAG CTA AAT AAA GAC ATG ACC TAT GCG
 Asn Arg Tyr His Pro Leu Gly Gln Leu Asn Lys Asp Met Thr Tyr Ala
 1592 1601 1610 1619 1628
 CAG TTG TAC TCC TTC CTG AAC TGC CTC CAG GTC AGC CCC TGC CAC GGC
 Gln Leu Tyr Ser Phe Leu Asn Cys Leu Gln Val Ser Pro Cys His Gly
 1638 1646 1655 1664 1673 1682
 TGG ATG TCT TCC AAC AAG ACG TTG CGG ACT CTC ACT TCA GAG AGA GCA
 Trp Met Ser Ser Asn Lys Thr Leu Arg Thr Leu Thr Ser Glu Arg Ala
 1691 1700 1709 1718 1727
 GAG CAA CTC TCC AAC ACA CTG AAA AAA ATT GCA GCC AGT GAG AAA TTT
 Glu Gln Leu Ser Asn Thr Leu Lys Lys Ile Ala Ala Ser Glu Lys Phe
 1736 1745 1754 1763 1772
 ACA AAC TTC AAT CTT TTC TAC ATG GAT TTT GCC TTC CAT GAA ATC ATA
 Thr Asn Phe Asn Leu Phe Tyr Met Asp Phe Ala Phe His Glu Ile Ile
 1782 1790 1799 1808 1817 1826
 CAG GAG TGG CAG AAG AGA GGC GGA CAG CCC TGG CAG CTC ATC GAG CCC
 Gln Glu Trp Gln Lys Arg Gly Gly Gln Pro Trp Gln Leu Ile Glu Pro
 1835 1844 1853 1862 1871
 GTG GAT GGA TTC CAC CCC AAC GAG GTG GCT TTG CTG TTG TTG GCG GAT
 Val Asp Gly Phe His Pro Asn Glu Val Ala Leu Leu Leu Leu Ala Asp
 1880 1889 1898 1907 1916
 CAT TTC TGG AAA AAG GTG CAG CTC CAG TGG CCC CAA ATC CTG GGA AAG
 His Phe Trp Lys Lys Val Gln Leu Gln Trp Pro Gln Ile Leu Gly Lys
 1926 1934 1943 1952 1961 1970
 GAG AAT CCG TTC AAC CCC CAG ATT AAA CAG GTG TTT GGA GAC CAA GGC
 Glu Asn Pro Phe Asn Pro Gln Ile Lys Gln Val Phe Gly Asp Gln Gly
 1979 1989 1999 2009 2019
 GGG CAC TGAGCCTCTC AGGAGCATGC ACCCCTGGGG AGCACAGGGA GGCAGAGGCT
 Gly His
 2020 2039 2049 2059 2069 2079
 TGGGTAACT CATTCCACAA ACCCTATGGG GGCTGCCACG TCACAGGCC CC AAAGGACTCT
 2090 2099 2109 2119 2129 2139
 TCTTCAGCAG CATCTTTGCA AAATGTCTTT CTCTCAATGA AGAGCATATC TGGACGACTG
 2150 2159
 TGCAATGCTG TGTGCTC

FIG. 2C.

SUBSTITUTE SHEET

10 20 30 40 50 60
 GAATTCGCGG CCGCAGAACC GCACACCACA GACTCCCTCC AGCTCTTTGT GTGTGGCTCT
 70 80 90 100 110 120
 CTCAGGGTCC AACAAGAGCA AGCTGTGGGT CTGTGAGTGT TTATGTGTGC TTTTATTAC
 130 140 150 160 170 180
 TTCACACTTA TTGAAAAGTG TGTATGTGAG AGGGTGGGGT GTGTGTGTCA AAGAGAGTGA
 190 200 210 220 230 240
 GGAAGAGAAG GAGAGAGAGA TCAATTGATT CTGCAGCCTC AGCTCCAGCA TCCCTCAGTT
 250 260 270 280 289
 GGGAGCTTCC AAAGCCGGGT GATCACTTGG GGTGCATAGC TCGGAG ATG CAG TCC
 Met Gln Ser
 298 307 316 325 334 343
 CCC TGG AAA ATC CTT ACG GTG GCG CCT CTA TTC TTG CTC CTG TCT CTT
 Pro Trp Lys Ile Leu Thr Val Ala Pro Leu Phe Leu Leu Leu Ser Leu
 352 361 370 379 388
 CAG TCC TCG GCC TCT CCA GCC AAC GAT GAC CAG TCC AGG CCC AGC CTC
 Gln Ser Ser Ala Ser Pro Ala Asn Asp Asp Gln Ser Arg Pro Ser Leu
 397 406 415 424 433
 TCG AAT GGG CAC ACC TGT GTA GGG TGT GTG CTG GTG GTG TCT GTA ATA
 Ser Asn Gly His Thr Cys Val Gly Cys Val Leu Val Val Ser Val Ile
 442 451 460 469 478 487
 GAA CAG CTT GCT CAA GTT CAC AAC TCG ACG GTC CAG GCC TCG ATG GAG
 Glu Gln Leu Ala Gln Val His Asn Ser Thr Val Gln Ala Ser Met Glu
 496 505 514 523 532
 AGA CTG TGC AGC TAC CTG CCT GAA AAA CTG TTC TTG AAA ACC ACC TGC
 Arg Leu Cys Ser Tyr Leu Pro Glu Lys Leu Phe Leu Lys Thr Thr Cys
 541 550 559 568 577
 TAT TTA GTC ATT GAC AAG TTT GGA TCA GAC ATC ATA AAA CTG CTT AGC
 Tyr Leu Val Ile Asp Lys Phe Gly Ser Asp Ile Ile Lys Leu Leu Ser
 586 595 604 613 622 631
 GCA GAT ATG AAT GCT GAT GTG GTA TGT CAC ACT CTG GAG TTT TGT AAA
 Ala Asp Met Asn Ala Asp Val Val Cys His Thr Leu Glu Phe Cys Lys
 640 649 658 667 676
 CAG AAC ACT GGC CAA CCA TTG TGT CAT CTC TAC CCT CTT CCC AAG GAG
 Gln Asn Thr Gly Gln Pro Leu Cys His Leu Tyr Pro Leu Pro Lys Glu
 685 694 703 712 721
 ACA TGG AAA TTT ACA CTA CAG AAG GCA AGA CAA ATT GTC AAG AAG TCC
 Thr Trp Lys Phe Thr Leu Gln Lys Ala Arg Gln Ile Val Lys Lys Ser
 730 739 748 757 766 775
 CCG ATT CTG AAA TAT TCT AGA AGT GGT TCT GAC ATT TGT TCA CTC CCG
 Pro Ile Leu Lys Tyr Ser Arg Ser Gly Ser Asp Ile Cys Ser Leu Pro

FIG. 3A.

SUBSTITUTE SHEET

784	793	802	811	820	
GTT TTG GCC AAG ATC TGC CAG AAA ATT AAA TTA GCT ATG GAA CAG TCT					
Val Leu Ala Lys Ile Cys Gln Lys Ile Lys Leu Ala Met Glu Gln Ser					
829	838	847	856	865	
GTG CCA TTC AAA GAT GTG GAT TCA GAC AAA TAC AGC GTT TTC CCA ACA					
Val Pro Phe Lys Asp Val Asp Ser Asp Lys Tyr Ser Val Phe Pro Thr					
874	883	892	901	910	919
CTG CGG GGC TAT CAC TGG CGG GGG AGA GAC TGT AAT GAC AGC GAC GAG					
Leu Arg Gly Tyr His Trp Arg Gly Arg Asp Cys Asn Asp Ser Asp Glu					
928	937	946	955	964	
TCA GTG TAC CCA GGT AGA AGG CCG AAC AAC TGG GAT GTC CAT CAG GAT					
Ser Val Tyr Pro Gly Arg Arg Pro Asn Asn Trp Asp Val His Gln Asp					
973	982	991	1000	1009	
TCA AAC TGT AAT GGC ATT TGG GGT GTC GAT CCA AAA GAT GGA GTT CCA					
Ser Asn Cys Asn Gly Ile Trp Gly Val Asp Pro Lys Asp Gly Val Pro					
1019	1027	1036	1045	1054	1063
TAT GAG AAG AAA TTC TGT GAA GGT TCA CAG CCC AGG GGA ATC ATT TTG					
Tyr Glu Lys Lys Phe Cys Glu Gly Ser Gln Pro Arg Gly Ile Ile Leu					
1072	1081	1090	1099	1108	
CTG GGA GAC TCA GCT GGG GCT CAT TTT CAC ATC TCT CCT GAA TGG ATC					
Leu Gly Asp Ser Ala Gly Ala His Phe His Ile Ser Pro Glu Trp Ile					
1117	1126	1135	1144	1153	
ACA GCG TCG CAG ATG TCT TTG AAC TCT TTC ATC AAT CTA CCA ACA GCC					
Thr Ala Ser Gln Met Ser Leu Asn Ser Phe Ile Asn Leu Pro Thr Ala					
1163	1171	1180	1189	1198	1207
CTT ACC AAC GAG CTT GAC TGG CCC CAA CTC TCT GGT GCT ACA GGA TTT					
Leu Thr Asn Glu Leu Asp Trp Pro Gln Leu Ser Gly Ala Thr Gly Phe					
1216	1225	1234	1243	1252	
CTG GAC TCC ACT GTT GGA ATT AAA GAA AAA TCT ATT TAC CTT CGC TTA					
Leu Asp Ser Thr Val Gly Ile Lys Glu Lys Ser Ile Tyr Leu Arg Leu					
1261	1270	1279	1288	1297	
TGG AAA AGA AAC CAC TGT AAT CAC AGG GAC TAC CAG AAT ATT TCA AGA					
Trp Lys Arg Asn His Cys Asn His Arg Asp Tyr Gln Asn Ile Ser Arg					
1307	1315	1324	1333	1342	1351
AAT GGT GCA TCT TCC CGA AAC CTG AAG AAA TTT ATA GAA AGC TTG TCT					
Asn Gly Ala Ser Ser Arg Asn Leu Lys Lys Phe Ile Glu Ser Leu Ser					
1360	1369	1378	1387	1396	
AGA AAC AAG GTG TTG GAC TAT CCC GCC ATC GTT ATA TAT GCC ATG ATT					
Arg Asn Lys Val Leu Asp Tyr Pro Ala Ile Val Ile Tyr Ala Met Ile					
1405	1414	1423	1432	1441	
GGA AAT GAT GTC TGC AGT GGG AAG AGT GAC CCA GTC CCA GCC ATG ACC					
Gly Asn Asp Val Cys Ser Gly Lys Ser Asp Pro Val Pro Ala Met Thr					

FIG. 3B.**SUBSTITUTE SHEET**

1451 1459 1468 1477 1486 1495
 ACT CCT GAG AAA CTC TAC TCC AAC GTC ATG CAG ACT CTG AAG CAT CTA
 Thr Pro Glu Lys Leu Tyr Ser Asn Val Met Gln Thr Leu Lys His Leu

1504 1513 1522 1531 1540
 AAT TCC CAC CTG CCC AAT GGC AGC CAT GTT ATT TTG TAT GGC TTA CCA
 Asn Ser His Leu Pro Asn Gly Ser His Val Ile Leu Tyr Gly Leu Pro

1549 1558 1567 1576 1585
 GAT GGA ACC TTT CTC TGG GAT AAT TTG CAC AAC AGA TAT CAT CCT CTC
 Asp Gly Thr Phe Leu Trp Asp Asn Leu His Asn Arg Tyr His Pro Leu

1595 1603 1612 1621 1630 1639
 GGC CAG CTA AAT AAA GAC ATG ACC TAT GCG CAG TTG TAC TCC TTC CTG
 Gly Gln Leu Asn Lys Asp Met Thr Tyr Ala Gln Leu Tyr Ser Phe Leu

1648 1657 1666 1675 1684
 AAC TGC CTC CAG GTC AGC CCC TGC CAC GGC TGG ATG TCT TCC AAC AAG
 Asn Cys Leu Gln Val Ser Pro Cys His Gly Trp Met Ser Ser Asn Lys

1693 1702 1711 1720 1729
 ACG TTG CGG ACT CTC ACT TCA GAG AGA GCA GAG CAA CTC TCC AAC ACA
 Thr Leu Arg Thr Leu Thr Ser Glu Arg Ala Glu Gln Leu Ser Asn Thr

1739 1747 1756 1765 1774 1783
 CTG AAA AAA ATT GCA GCC AGT GAG AAA TTT ACA AAC TTC AAT CTT TTC
 Leu Lys Lys Ile Ala Ala Ser Glu Lys Phe Thr Asn Phe Asn Leu Phe

1792 1801 1810 1819 1828
 TAC ATG GAT TTT GCC TTC CAT GAA ATC ATA CAG GAG TGG CAG AAG AGA
 Tyr Met Asp Phe Ala Phe His Glu Ile Ile Gln Glu Trp Gln Lys Arg

1837 1846 1855 1864 1873
 GGC GGA CAG CCC TGG CAG CTC ATC GAG CCC GTG GAT GGA TTC CAC CCC
 Gly Gly Gln Pro Trp Gln Leu Ile Glu Pro Val Asp Gly Phe His Pro

1883 1891 1900 1909 1918 1927
 AAC GAG GTG GCT TTG CTG TTG TTG GCG GAT CAT TTC TGG AAA AAG GTG
 Asn Glu Val Ala Leu Leu Leu Leu Ala Asp His Phe Trp Lys Lys Val

1936 1945 1954 1963 1972
 CAG CTC CAG TGG CCC CAA ATC CTG GGA AAG GAG AAT CCG TTC AAC CCC
 Gln Leu Gln Trp Pro Gln Ile Leu Gly Lys Glu Asn Pro Phe Asn Pro

1981 1990 1999 2008 2014
 CAG ATT AAA CAG GTG TTT GGA GAC CAA GGC GGG CAC TGA GCCTCTC
 Gln Ile Lys Gln Val Phe Gly Asp Gln Gly Gly His

2024 2034 2044 2054 2064 2074
 AGGAGCATGC ACCCCTGGGG AGCACAGGGA GGCAGAGGCT TGGGTAAACT CATTCCACAA

2084 2094 2104 2114 2124 2134
 ACCCTATGGG GGCTGCCACG TCACAGGCC AAAGGACTCT TCTTCAGCAG CATCTTTGCA

2144 2154 2164 2174 2184 2194
 AAATGTCTTT CTCTCAATGA AGAGCATATC TGGACGACTG TGCAATGCTG TGTGCTCCCG

FIG. 3C.

SUBSTITUTE SHEET

2204	2214	2224	2234	2244	2254
GGATCAGTAA	CCCTTCCGCT	GTTCTGAAA	TAACCTTTCA	TAAAGTGCTT	TGGGTGCCAT
2264	2274	2284			
TCCAAAAAAA	AAAAAAAAAA	AAAAAAAAAA			

FIG. 3D.

										10											20											29											38											47											
										GAATTCGTCG											ACCACC											ATG CAG TCC CCC TGG AAA ATC CTT ACG GTG GCG																																	
																																Met Gln Ser Pro Trp Lys Ile Leu Thr Val Ala																																	
										56											65											74											83											92											
										CCT CTA TTC TTG CTC CTG TCT CTT CAG TCC TCG GCC TCT CCA GCC AAC																																																							
										Pro Leu Phe Leu Leu Leu Ser Leu Gln Ser Ser Ala Ser Pro Ala Asn																																																							
										101											110											119											128											137											146
										GAT GAC CAG TCC AGG CCC AGC CTC TCG AAT GGG CAC ACC TGT GTA GGG																																																							
										Asp Asp Gln Ser Arg Pro Ser Leu Ser Asn Gly His Thr Cys Val Gly																																																							
										155											164											173											182											191											
										TGT GTG CTG GTG GTG TCT GTA ATA GAA CAG CTT GCT CAA GTT CAC AAC																																																							
										Cys Val Leu Val Val Ser Val Ile Glu Gln Leu Ala Gln Val His Asn																																																							
										200											209											218											227											236											
										TCG ACG GTC CAG GCC TCG ATG GAG AGA CTG TGC AGC TAC CTG CCT GAA																																																							
										Ser Thr Val Gln Ala Ser Met Glu Arg Leu Cys Ser Tyr Leu Pro Glu																																																							
										245											254											263											272											281											290
										AAA CTG TTC TTG AAA ACC ACC TGC TAT TTA GTC ATT GAC AAG TTT GGA																																																							
										Lys Leu Phe Leu Lys Thr Thr Cys Tyr Leu Val Ile Asp Lys Phe Gly																																																							
										299											208											317											326											335											
										TCA GAC ATC ATA AAA CTG CTT AGC GCA GAT ATG AAT GCT GAT GTG GTA																																																							
										Ser Asp Ile Ile Lys Leu Leu Ser Ala Asp Met Asn Ala Asp Val Val																																																							
										344											353											362											371											380											
										TGT CAC ACT CTC GAG TTT TGT AAA CAG AAC ACT GGC CAA CCA TTG TGT																																																							
										Cys His Thr Leu Glu Phe Cys Lys Gln Asn Thr Gly Gln Pro Leu Cys																																																							
										389											398											407											416											425											434
										CAT CTC TAC CCT CTT CCC AAG GAG ACA TGG AAA TTT ACA CTA CAG AAG																																																							
										His Leu Tyr Pro Leu Pro Lys Glu Thr Trp Lys Phe Thr Leu Gln Lys																																																							
										443											452											461											470											479											
										GCA AGA CAA ATT GTC AAG AAG TCC CCG ATT CTG AAA TAT TCT AGA AGT																																																							
										Ala Arg Gln Ile Val Lys Lys Ser Pro Ile Leu Lys Tyr Ser Arg Ser																																																							
										488											497											506											515											524											
										GGT TCT GAC ATT TGT TCA CTC CCG GTT TTG GCC AAG ATC TGC CAG AAA																																																							
										Gly Ser Asp Ile Cys Ser Leu Pro Val Leu Ala Lys Ile Cys Gln Lys																																																							
										533											542											551											560											569											578
										ATT AAA TTA GCT ATG GAA CAG TCT GTG CCA TTC AAA GAT GTG GAT TCA																																																							
										Ile Lys Leu Ala Met Glu Gln Ser Val Pro Phe Lys Asp Val Asp Ser																																																							
										587											596											605											614											623											
										GAC AAA TAC AGC GTT TTC CCA ACA CTG CGG GGC TAT CAC TGG CGG GGG																																																							
										Asp Lys Tyr Ser Val Phe Pro Thr Leu Arg Gly Tyr His Trp Arg Gly																																																							
										632											641											650											659											668											
										AGA GAC TGT AAT GAC AGC GAC GAG TCA GTG TAC CCA GGT AGA AGG CCG																																																							
										Arg Asp Cys Asn Asp Ser Asp Glu Ser Val Tyr Pro Gly Arg Arg Pro																																																							

FIG. 4A.

SUBSTITUTE SHEET

677 AAC Asn	AAC Asn	TGG Trp	686 GAT Asp	GTC Val	CAT His	695 CAG Gln	GAT Asp	TCA Ser	704 AAC Asn	TGT Cys	AAT Asn	713 GGC Gly	ATT Ile	TGG Trp	722 GGT Gly
GTC Val	GAT Asp	731 CCA Pro	AAA Lys	GAT Asp	740 GGA Gly	GTT Val	CCA Pro	747 TAT Tyr	GAG Glu	AAG Lys	758 AAA Lys	TTC Phe	TGT Cys	767 GAA Glu	GGT Gly
TCA Ser	776 CAG Gln	CCC Pro	AGG Arg	785 GGA Gly	ATC Ile	ATT Ile	794 TTG Leu	CTG Leu	803 GGA Gly	TCA Ser	GCT Ala	812 GGG Gly	GCT Ala	CAT His	
821 TTT Phe	CAC His	ATC Ile	830 TCT Ser	CCT Pro	GAA Glu	TGG Trp	ATC Ile	ACA Thr	848 GCG Ala	TCG Ser	CAG Gln	857 ATG Met	TCT Ser	TTG Leu	866 AAC Asn
TCT Ser	875 TTC Phe	ATC Ile	AAT Asn	CTA Leu	884 CCA Pro	ACA Thr	GCC Ala	893 CTT Leu	ACC Thr	AAC Asn	902 GAG Glu	CTT Leu	GAC Asp	911 TGG Trp	CCC Pro
CAA Gln	920 CTC Leu	TCT Ser	GGT Gly	929 GCT Ala	ACA Thr	GGA Gly	938 TTT Phe	CTG Leu	GAC Asp	947 TCC Ser	ACT Thr	GTT Val	956 GGA Gly	ATT Ile	AAA Lys
965 GAA Glu	AAA Lys	TCT Ser	974 ATT Ile	TAC Tyr	CTT Leu	CGC Arg	TTA Leu	TGG Trp	992 AAA Lys	AGA Arg	AAC Asn	1001 CAC His	TGT Cys	AAT Asn	1010 CAC His
AGG Arg	1019 GAC Asp	TAC Tyr	CAG Gln	AAT Asn	1028 ATT Ile	TCA Ser	AGA Arg	AAT Asn	1037 GGT Gly	GCA Ala	TCT Ser	TCC Ser	CGA Arg	AAC Asn	1055 CTG Leu
AAG Lys	1064 AAA Lys	TTT Phe	ATA Ile	GAA Glu	1073 AGC Ser	TTG Leu	TCT Ser	AGA Arg	AAC Asn	1091 AAG Lys	GTG Val	TTG Leu	GAC Asp	TAT Tyr	CCC Pro
1100 GCC Ala	ATC Ile	GTT Val	1118 ATA Ile	TAT Tyr	GCC Ala	ATG Met	ATT Ile	GGA Gly	1136 AAT Asn	GAT Asp	1145 GTC Val	TGC Cys	AGT Ser	GGG Gly	1154 AAG Lys
AGT Ser	1163 GAC Asp	CCA Pro	GTC Val	CCA Pro	1172 GCC Ala	ATG Met	ACC Thr	ACT Thr	1181 CCT Pro	GAG Glu	1190 AAA Lys	CTC Leu	TAC Tyr	TCC Ser	1199 AAC Asn
GTC Val	1208 ATG Met	CAG Gln	ACT Thr	1217 CTG Leu	AAG Lys	CAT His	CTA Leu	AAT Asn	1226 TCC Ser	CAC His	CTG Leu	CCC Pro	AAT Asn	GGA Gly	1244 TCC Ser
CAT His	1254 GTT Val	ATT Ile	1262 TTG Leu	TAT Tyr	GGC Gly	1271 TTA Leu	CCA Pro	GAT Asp	1280 GGA Gly	ACC Thr	TTT Phe	CTC Leu	TGG Trp	GAT Asp	1298 AAT Asn
TTG Leu	1307 CAC His	AAC Asn	AGA Arg	TAT Tyr	1316 CAT His	CCT Pro	CTC Leu	GGC Gly	1325 CAG Gln	CTA Leu	AAT Asn	1334 AAA Lys	GAC Asp	ATG Met	1343 ACC Thr

FIG. 4B.

SUBSTITUTE SHEET

1352 1361 1370 1379 1388
 TAT GCG CAG TTG TAC TCC TTC CTG AAC TGC CTC CAG GTC AGC CCC TGC
 Tyr Ala Gln Leu Tyr Ser Phe Leu Asn Cys Leu Gln Val Ser Pro Cys

 1398 1406 1415 1424 1433 1442
 CAC GGC TGG ATG TCT TCC AAC AAG ACG TTG CGG ACT CTC ACT TCA GAG
 His Gly Trp Met Ser Ser Asn Lys Thr Leu Arg Thr Leu Thr Ser Glu

 1451 1460 1469 1478 1487
 AGA GCA GAG CAA CTC TCC AAC ACA CTG AAA AAA ATT GCA GCC AGT GAG
 Arg Ala Glu Gln Leu Ser Asn Thr Leu Lys Lys Ile Ala Ala Ser Glu

 1496 1505 1514 1523 1532
 AAA TTT ACA AAC TTC AAT CTT TTC TAC ATG GAT TTT GCC TTC CAT GAA
 Lys Phe Thr Asn Phe Asn Leu Phe Tyr Met Asp Phe Ala Phe His Glu

 1542 1550 1589 1568 1577 1586
 ATC ATA CAG GAG TGG CAG AAG AGA GGC GGA CAG CCC TGG CAG CTC ATC
 Ile Ile Gln Glu Trp Gln Lys Arg Gly Gly Gln Pro Trp Gln Leu Ile

 1595 1604 1613 1622 1631
 GAG CCC GTG GAT GGA TTC CAC CCC AAC GAG GTG GCT TTG CTG TTG TTG
 Glu Pro Val Asp Gly Phe His Pro Asn Glu Val Ala Leu Leu Leu Leu

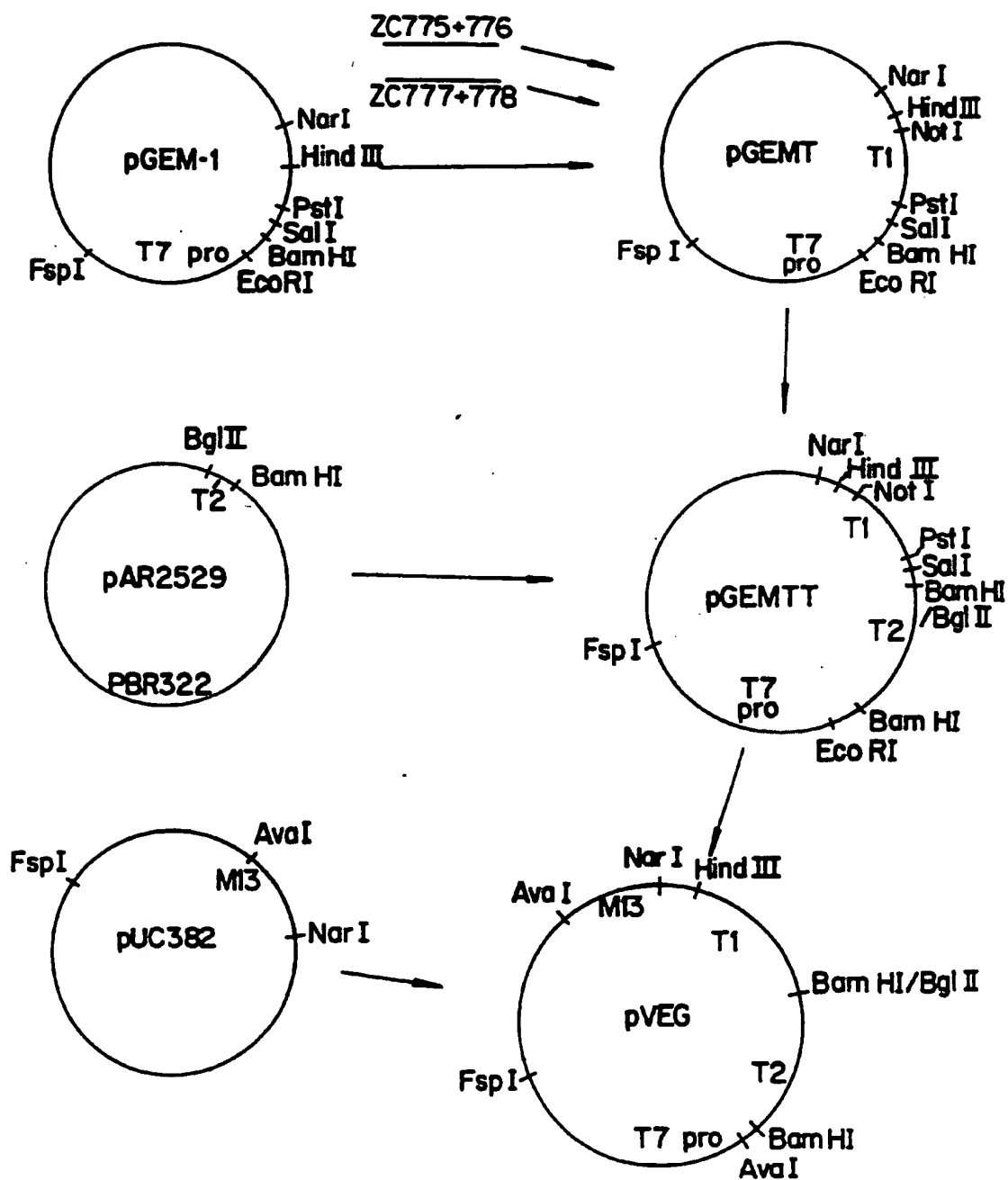
 1640 1649 1658 1667 1676
 GCG GAT CAT TTC TGG AAA AAG GTG CAG CTC CAG TGG CCC CAA ATC CTG
 Ala Asp His Phe Trp Lys Lys Val Gln Leu Gln Trp Pro Gln Ile Leu

 1686 1694 1703 1712 1721 1730
 GGA AAG GAG AAT CCG TTC AAC CCC CAG ATT AAA CAG GTG TTT GGA GAC
 Gly Lys Glu Asn Pro Phe Asn Pro Gln Ile Lys Gln Val Phe Gly Asp

 1739 1748 1757
 CAA GGC GGG CAC TGAGAATTCG TCGAC
 Gln Gly Gly His
 575

FIG. 4C.**SUBSTITUTE SHEET**

14 / 19

**FIG. 5.**

SUBSTITUTE SHEET

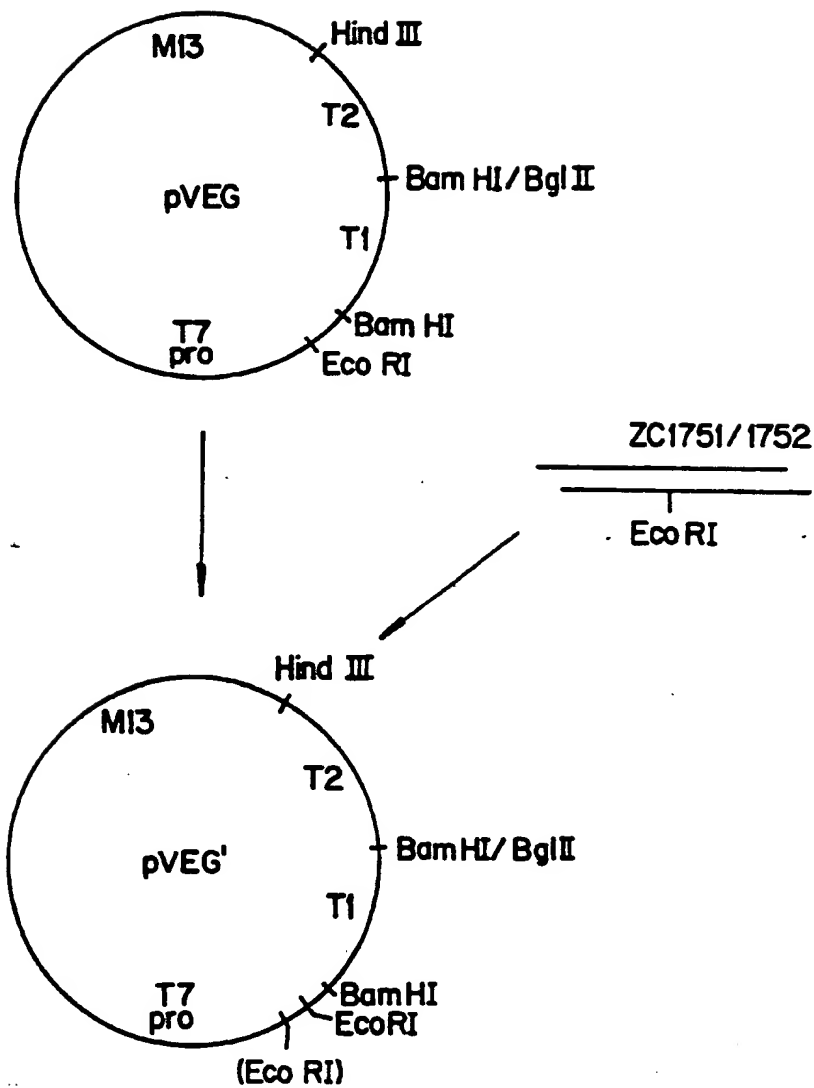


FIG. 6.

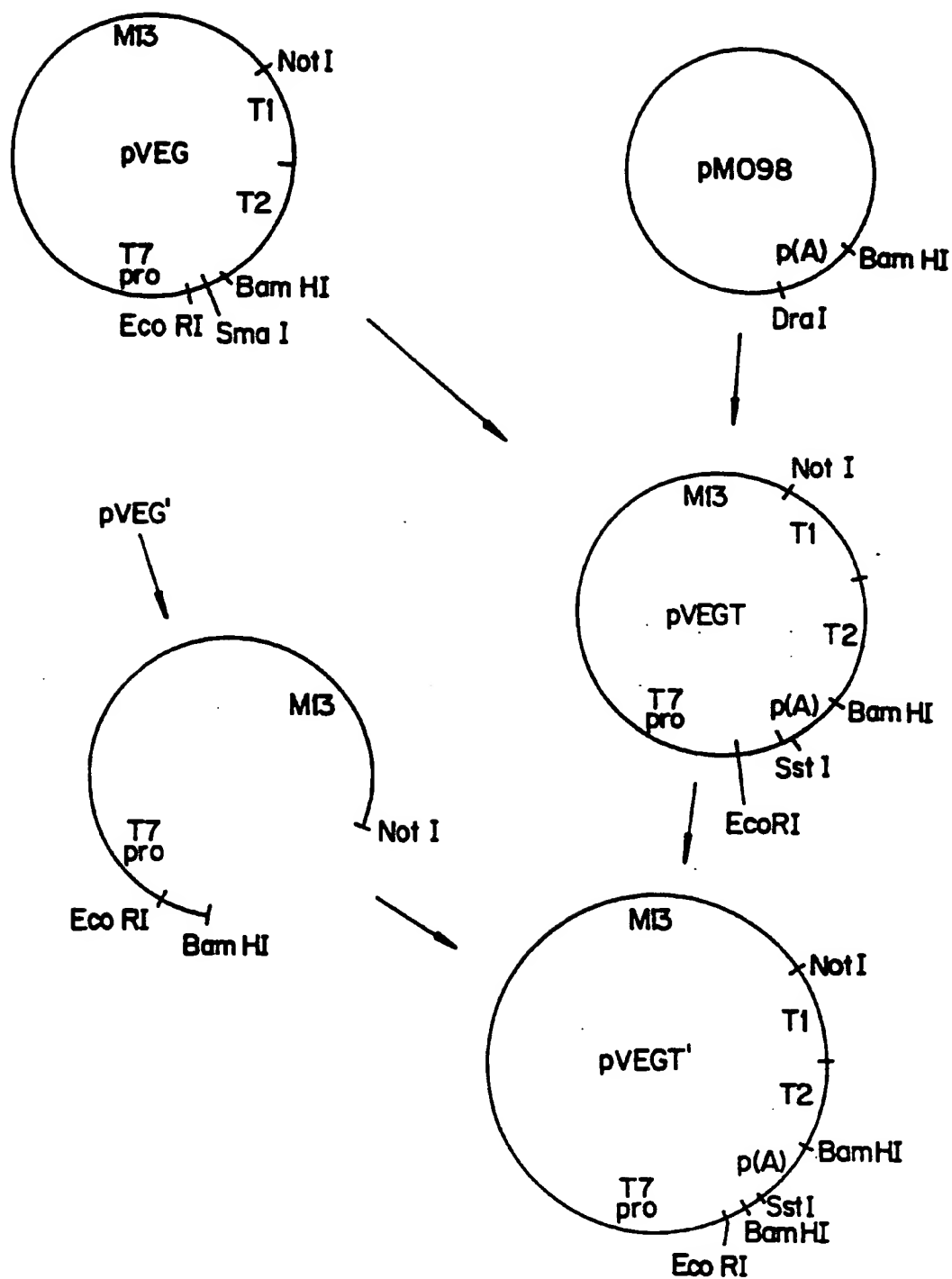


FIG. 7.

SUBSTITUTE SHEET

17 / 19

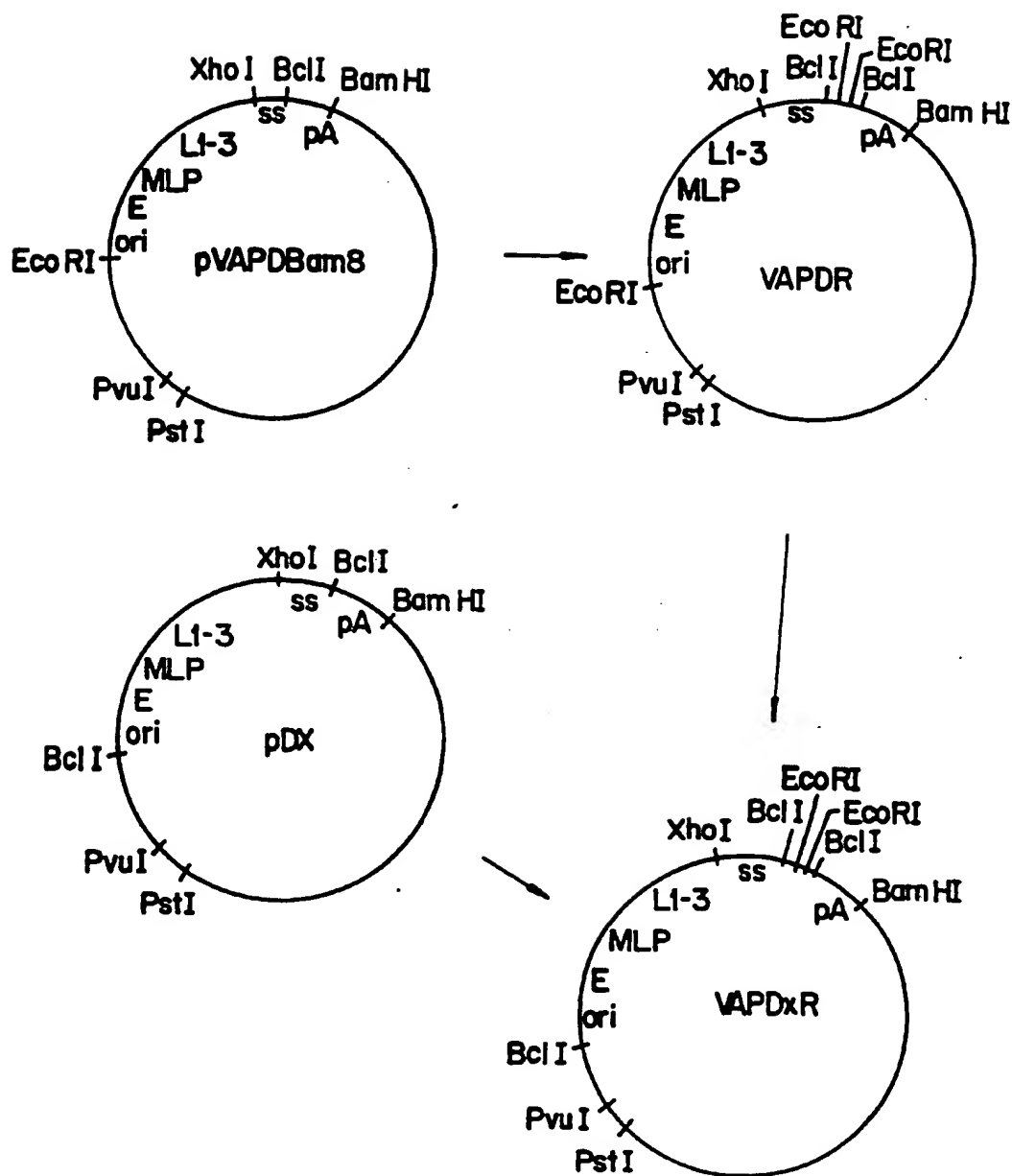


FIG. 8.

SUBSTITUTE SHEET

18 / 19

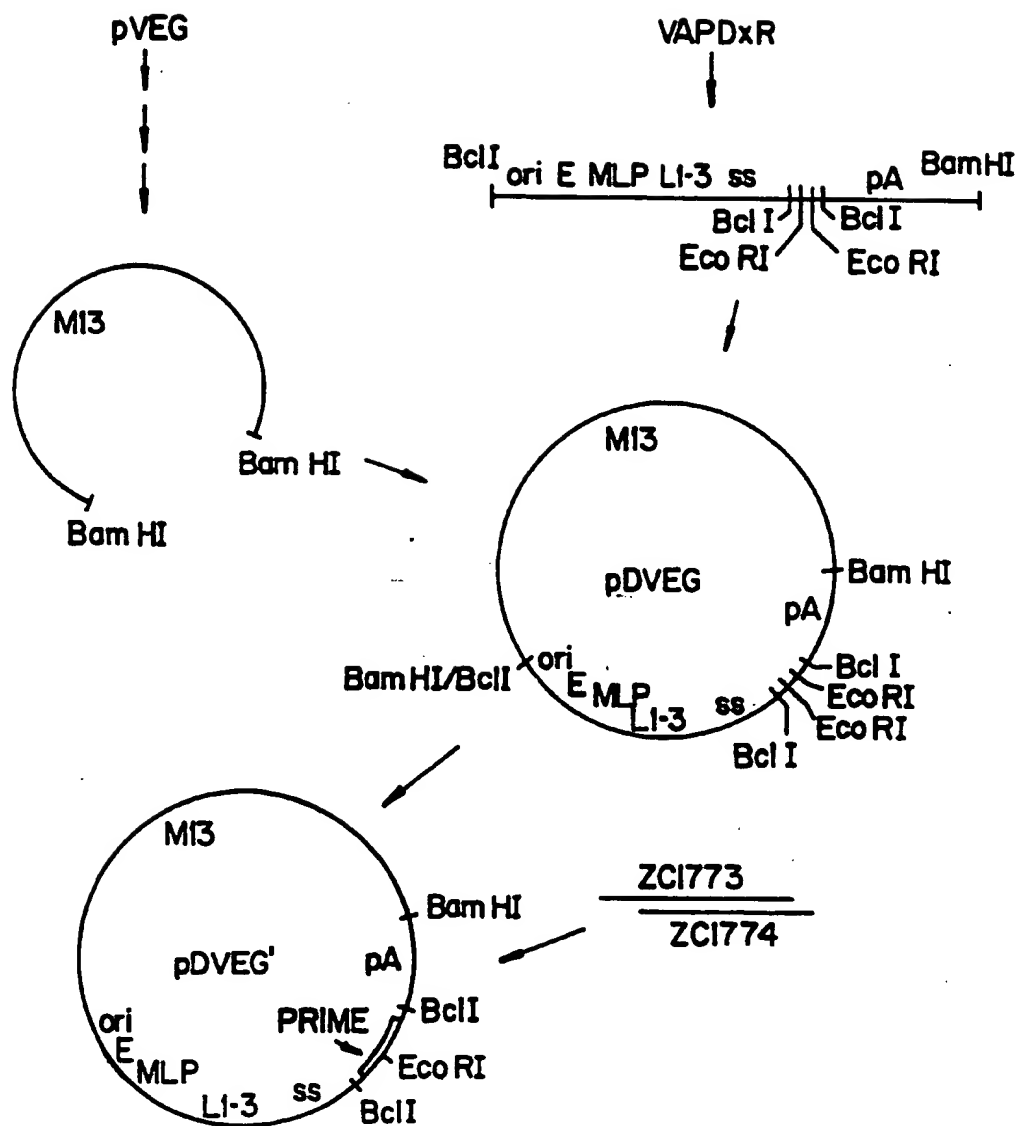


FIG. 9.

SUBSTITUTE SHEET

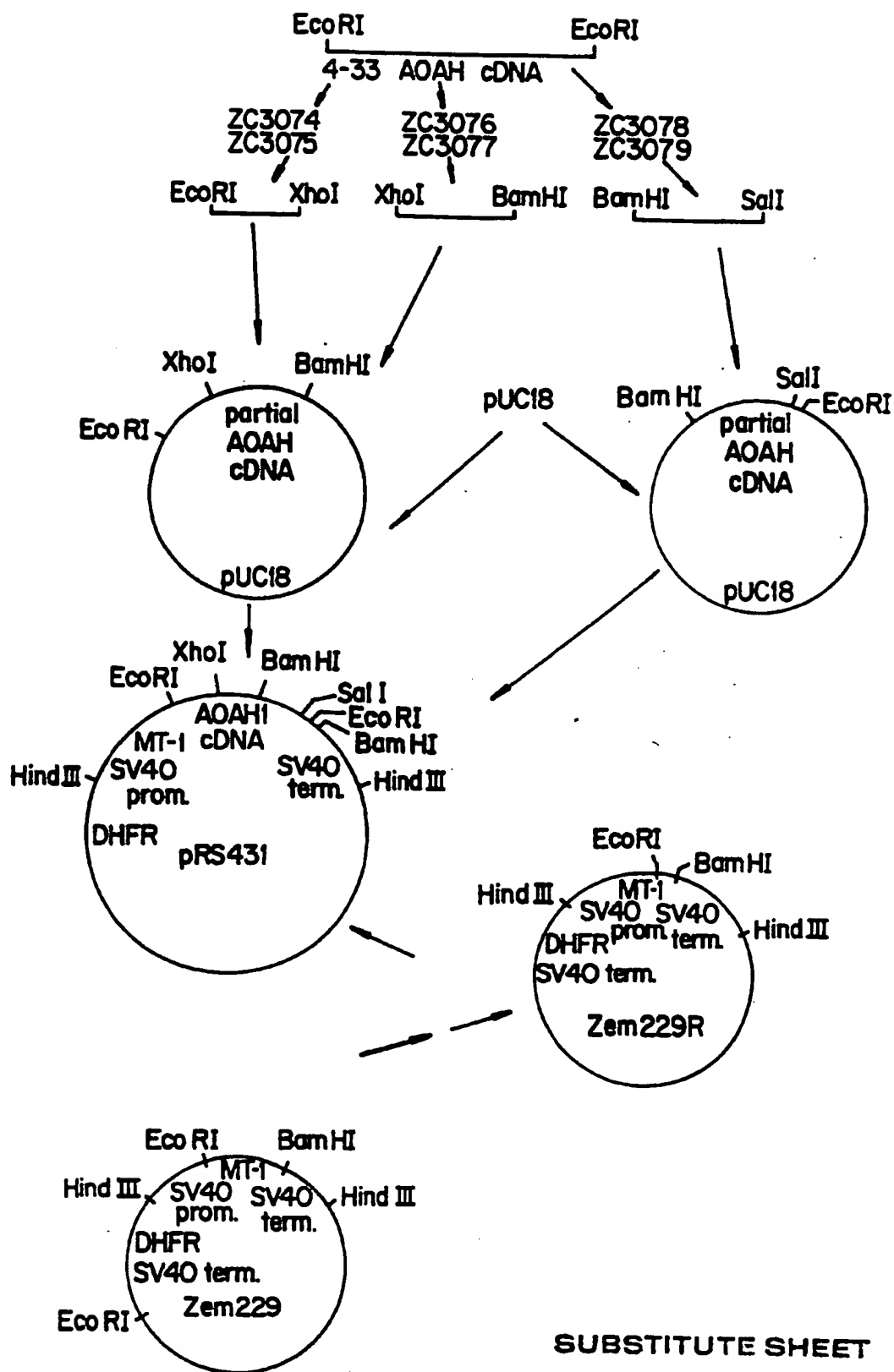


FIG. 10.

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/06569

I. CLASSIFICATION OF SUBJECT MATTER (In several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC(5): C12N 15/00, 15/55, 15/79; A61K 37/54		
U.S. Cl.: 435/69.1, 232.5, 320.1; 536/27; 424/94.61		
II. FIELDS SEARCHED		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
U.S. Cl.	435/69.1, 232.5, 320.1; 536/27; 424/94.61	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched *		
ELECTRONIC DATABASES: APS, STN/CHEMICAL ABSTRACTS, DIALOG/BIOSIS		
III. DOCUMENTS CONSIDERED TO BE RELEVANT *		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. 13
Y, P	U.S.A. 5,013,661 (MUNFORD ET AL.) 07 MAY 1991. see columns 1-3.6-13, and Fig. 3.	1-4, 14-18, 20-21, 36-43, 49-52, 57
Y, P	U.S.A. 5,047,336 (CATE ET AL) 10 SEPTEMBER 1991. see column 2. line 60-column 4. line 10. and columns 12-22.	1-4, 14-16, 20-21, 36-38, 41-43, 49-52
Y, P	U.S.A. 5,037,743 (WELCH ET AL) 06 AUGUST 1991. see columns 3-27 and Figures 1-5, 9-11, 18-19.	1-4, 14-17, 20-21, 36-39, 41-43, 49-52
Y	U.S.A. 4,727,138 (GOEDDEL ET AL) 23 FEBRUARY 1988. see column 3-4, 9-10, 17-22. and Fig. 16.	1-4, 14-16, 20-21, 36-38, 41-43, 49-52
Y	EP.A. 0,310,137 (WELCH ET AL) 05 APRIL 1989. see pages 3-20 and Figures 1-5, 9-11, 18-19.	1-4, 14-17, 20-21, 36-39, 41-43, 49-52
<p>* Special categories of cited documents: **</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another claim or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cite to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Making of this International Search Report
15 November 1991		15 JAN 1992
International Searching Authority		Signature of Authorized Officer
RO/US		William Moore

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 264, No. 26, issued 15 September 1989. Munford et al. "Purification of Acylglycerol Hydrolase, a Leukocyte Enzyme That Removes Secondary Acyl Chains from Bacterial Lipopolysaccharides". pages 15613-15619. see entire document.	1-4.14-18. 20-21.36-43. 49-52
Y	NUCLEIC ACIDS RESEARCH. Vol. 17, No. 8. issued 04 April 1989. Belyavsky et al. "PCR-Based cDNA Library Construction: General cDNA Libraries at the Level of a Few Cells". pages 2919-2932. see entire article.	1-4.14-18. 20-21.36-43. 49-52
Y	JOURNAL OF EXPERIMENTAL MEDICINE, Vol. 152, issued October 1980, Mellman et al. "Purification of a Functional Mouse Fc Receptor Through the Use of a Monoclonal Antibody". pages 1048-1069. see pages 1049-1052. 1058-1059	1-4.14-18. 20-21.36-43. 49-52